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# THE EFFECT OF STIMULATION OF EXTRINSIC NERVES ON PERISTALSIS AND ON THE RELEASE OF 5-HYDROXY-TRYPTAMINE IN THE LARGE INTESTINE OF THE GUINEA-PIG AND OF THE RABBIT

By C. Y. LEE*

*From the Department of Pharmacology, University of Oxford*

(*Received* 16 *October* 1959)

Since the classical work by Trendelenburg (1917), many studies have been published on the peristaltic activity of the small intestine (for references see Bülbring & Lin, 1958). In a recent investigation Bülbring & Lin (1958) found that small amounts of 5-hydroxytryptamine (5-HT) were continuously released into the lumen of an isolated loop of ileum and that 5-HT, if applied intraluminally, stimulated peristalsis by its action on sensory receptors deeply seated in the intestinal mucosa. These findings have been extended by experiments on small intestine *in situ* (Bülbring & Crema, 1958, 1959*a*, *b*), but little is known about the effect of 5-HT on, and the release of 5-HT from, the large intestine. Moreover it is not known whether the release of 5-HT from the intestine is controlled by the extrinsic nerves. Bayliss & Starling (1900) have described the movements of the large intestine *in situ* and the effects of stimulating its sympathetic and parasympathetic nerve supply. Since the distal colon can be excised with both the lumbar sympathetic outflow and the pelvic nerve attached (Garry & Gillespie, 1954, 1955), it seemed of interest to study the action of 5-HT on the peristalsis of the isolated colon and to investigate the effect of extrinsic nerve stimulation on 5-HT release.

The effects of atropine, of hexamethonium and of nicotine on the peristaltic reflex as well as on the extrinsic nerve stimulation were also investigated, in the hope of throwing some light on the relationship between the nervous pathways of the peristaltic reflex arc and those of the extrinsic innervation.

A preliminary account of some of the results has been given in a communication to the Physiological Society (Lee, 1959).

* On leave of absence from the Pharmacological Institute, National Taiwan University, Taipei, Taiwan (Formosa), China.

## METHODS

Guinea-pigs and rabbits of either sex were used. They were stunned and bled. Loops from the oral or caudal part of the distal colon were excised to record peristaltic activity; a few experiments were also done on isolated loops of ileum for comparison. A piece of intestine about 7–8 cm long was suspended at 37° ± 0·5° C in Tyrode solution (containing $MgCl_2$ 0·01 g/l.), which was continuously oxygenated with 95 % $O_2$ + 5 % $CO_2$. The solution in the 60 ml. organ bath was continuously changed by a slow flow of about 2 ml./min, the level being kept constant by overflow. Whenever a drug was added to the bath the flow was stopped in order to avoid dilution of the drug concentration. Oxygenated Tyrode solution from a Marriotte bottle flowed also through the lumen of the intestine.

Peristaltic activity was recorded by the method of Bülbring, Crema & Saxby (1958). This is so designed that peristaltic contractions are evoked at regular intervals, as fluid from a Marriotte bottle, kept at constant height, enters the lumen of the intestine at a slow rate. Each time the critical pressure is attained the fluid is expelled through a suspended U tube. This arrangement allows the longitudinal contractions to be recorded. The Marriotte bottle was kept 7–17 cm above bath level; the outflow tube only 3–9 cm, since it carried a rubber valve which added to the resistance and prevented fluid from siphoning over. Thus no fluid could escape except when it was expelled by peristaltic waves, which occurred usually once or twice per minute.

In some experiments, both outflow and inflow were recorded. For the outflow a volume recorder was used of the type described by Bülbring *et al.* (1958), each vertical stroke indicating expulsion of 1 ml. fluid. For the inflow a Thorpe drop recorder was arranged in such a way that the contact was broken at the moment when fluid was expelled—i.e. whenever a peristaltic wave occurred.

Preparations of the caudal part of the distal colon were dissected, with the extrinsic nerves, from rabbits and guinea-pigs according to the method of Garry & Gillespie (1954, 1955). The lumbar sympathetic outflow or the pelvic visceral nerve was stimulated by two irrigated electrodes (Burn & Rand, 1960) for 0·5 or 1 min with stimuli of 1–2 msec duration at a frequency of 10/sec from an electronic rectangular-pulse stimulator.

For the determination of 5-HT in the effluent, samples were collected into ice-cold test-tubes over periods of 3–10 min and immediately frozen in a deep-freeze. In the absence of peristalsis the effluent was obtained by lowering the resistance to the outflow, allowing a passive flow.

The 5-HT in the effluent was estimated by Vane's method (1957) on the rat stomach preparation in the presence of atropine $10^{-6}$ and mepyramine $10^{-7}$. Although this preparation is rather insensitive to histamine, the mepyramine was used to avoid errors due to large concentrations of histamine in the samples. The contractions produced by the samples were abolished by $10^{-5}$ BOL 148 (2-brom-D-lysergic acid diethylamide).

The following substances were used: atropine sulphate, hexamethonium bromide, nicotine hydrogen tartrate, 5-hydroxytryptamine-creatinine sulphate, adrenaline hydrochloride, mepyramine hydrochloride and BOL 148. Dosages refer to the salt, except for 5-HT and adrenaline.

## RESULTS

### *Normal propulsive activity of the colon*

The peristaltic waves of the isolated colon from the guinea-pig and from the rabbit were compared with those of the ileum in the same species, and Fig. 1 shows the following differences. First, the peristaltic wave of the colon was slower and more prolonged than that of the ileum, and the

emptying phase lasted 20–30 sec in the colon as against 5 sec in the ileum. Secondly, the longitudinal contractions were very regular in the ileum but rather irregular in the colon, in which there was no distinct 'preparatory

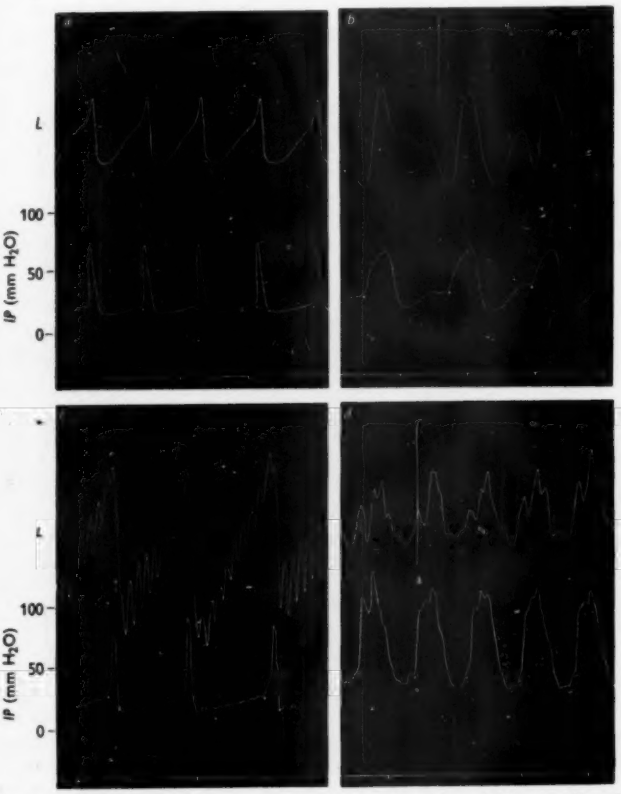


**Fig. 1.** Records of peristaltic waves of (*a*) isolated guinea-pig ileum, (*b*) guinea-pig colon, (*c*) rabbit ileum, (*d*) propulsive waves in rabbit colon. In this and all subsequent figures $L$ = longitudinal muscle contractions (upwards), $IP$ = intraluminal pressure (mm water) at oral end. The threshold at which the emptying phase starts is marked with dots. At this moment the pressure in the tube to the float recorder starts to rise sharply because the lumen is occluded, and drops again after the intraluminal contents have been expelled. Time marker, 30 sec.

phase'. Thirdly, the threshold pressure required to elicit the peristaltic reflex was higher for the colon than for the ileum, varying between 40 and 70 mm water.

The raised filling pressure was not the only stimulus to evoke peristalsis in the colon. In the preparation shown in Fig. 2*a* there were at first only rhythmic pendular movements but no propulsive contractions, although

the intraluminal pressure was high. The muscle coats then relaxed spontaneously, allowing considerable distension of the lumen, which was followed by a burst of propulsive waves. A similar phenomenon was described by Straub & Schild (1933) who found that the first response of the large intestine *in situ* to a raised filling pressure was 'diastole' and concluded that distension of the wall was essential for eliciting the reflex.

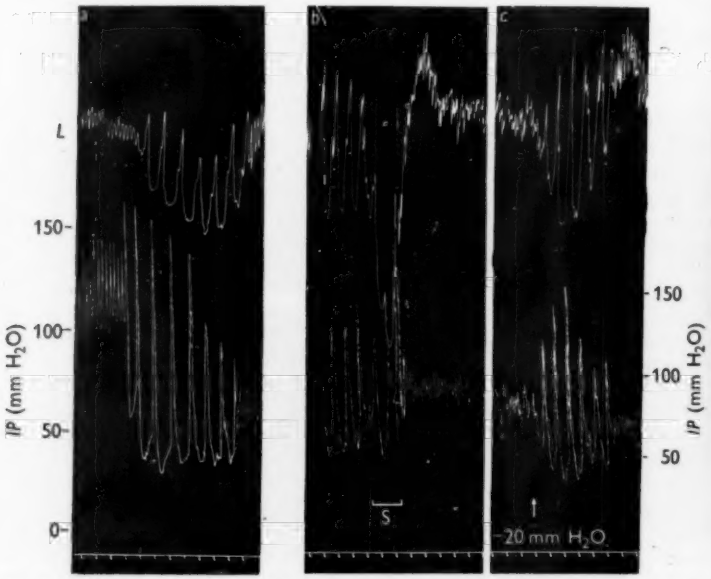


Fig. 2. Rabbit colon. Two different conditions preventing propulsive activity. (*a*) Pendular, non-propulsive movements at high intraluminal pressure during high tone of circular muscle; propulsive waves occurring only after spontaneous relaxation allowing distension of the lumen. (*b*) Different experiment: inhibition of propulsive activity due to excessive distension of lumen after sympathetic nerve stimulation at S. (*c*) 20 min after (*b*), reappearance of propulsive waves on lowering the filling pressure by 20 mm $H_2O$.

The behaviour shown in Fig. 2*a* in which groups of 5–10 propulsive waves alternated with periods of non-propulsive contractions occurred in a minority of the *in vitro* preparations. Usually it was possible to adjust the filling pressure in such a way that propulsive waves were evoked at fairly regular intervals for several hours. These were the conditions in which the effects of nerve stimulation and of various drugs were investigated.

*The effect of extrinsic nerve stimulation on propulsive activity*

Sympathetic nerve stimulation arrested propulsive activity. It caused an immediate relaxation of the longitudinal muscle in both species. The

response of the circular muscle was not constant; sometimes it relaxed immediately, but sometimes sympathetic stimulation caused an increase in the rate of circular contractions, or a sustained contraction. When sympathetic stimulation was stopped there followed, after a latent period of 5–30 sec, a transient increase in tone of both muscle coats; thereafter regular peristaltic activity was resumed. In some experiments, however, the lumen remained over-distended for some time and propulsive waves reappeared only when the filling pressure was lowered, as is shown in Fig. 2*b*, *c*.

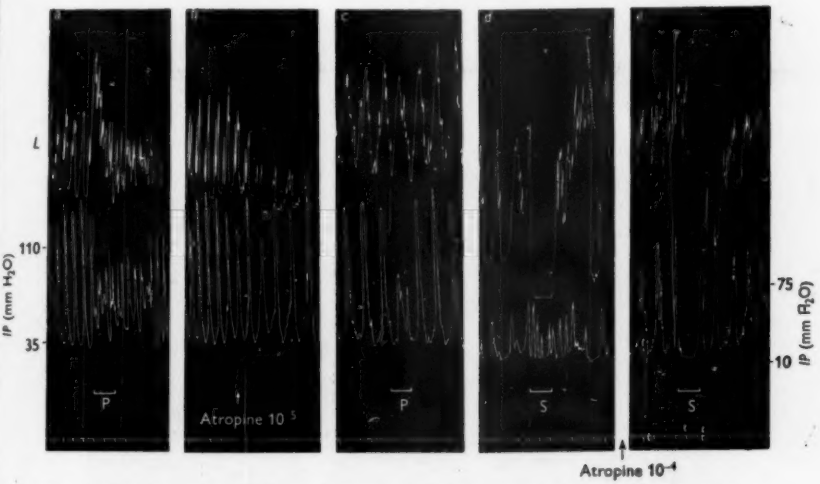


Fig. 3. Rabbit colon. Effect of atropine. Pelvic nerve stimulation (P) effective before atropine (*a*) but not after (*c*), while propulsive activity persists (*b*, *c*). Sympathetic stimulation (S), in another preparation, causing circular contractions before atropine (*d*) and relaxation after, but propulsive activity persists (*e*).

Pelvic nerve stimulation also arrested propulsive activity. It produced a sustained contraction of both muscle coats which stopped propulsive activity for the duration of the stimulus, and there was usually no increase in the number of propulsive waves afterwards.

### *Effect of atropine*

Atropine in concentrations as high as $10^{-4}$ did not abolish the propulsive activity in the rabbit colon. At first the tone of the longitudinal muscle decreased and the threshold pressure was slightly raised (Fig. 3*b*), but later the longitudinal muscle tone recovered and propulsive activity continued at a slightly slower rate (Fig. 3*c*). At the same time, however,

the response to pelvic nerve stimulation was abolished (Fig. 3*a*, *c*). When the concentration of atropine was raised to $10^{-4}$ propulsive activity persisted. Moreover, if the response of the circular muscle to sympathetic nerve stimulation was a contraction, this effect was abolished or reversed by atropine (Fig. 3*d*, *e*), but propulsive activity continued nevertheless. If a colon preparation showed irregular, non-propulsive contractions atropine converted these into regular, co-ordinated propulsive waves (Fig. 4*a*). The effect of atropine on the rabbit ileum was entirely different.

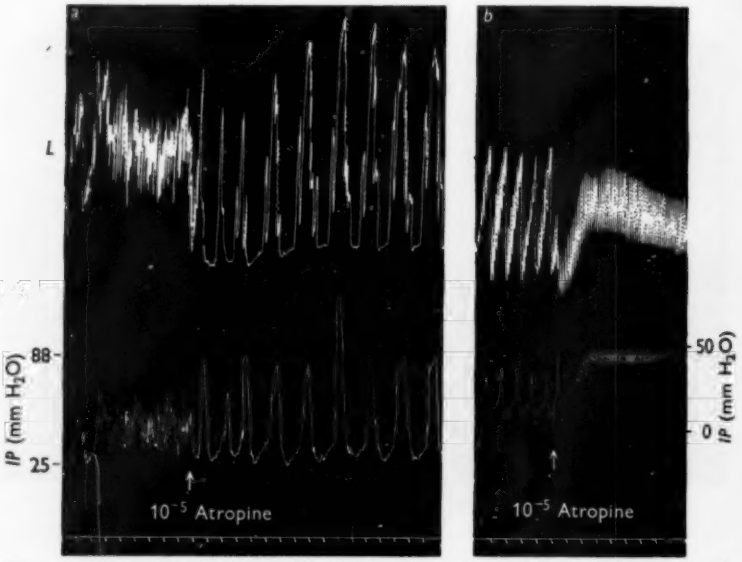


Fig. 4. Effect of atropine ($10^{-5}$) on propulsive activity of rabbit large (*a*) and small (*b*) intestine. (*a*) In the colon atropine abolished pendular activity and diminished longitudinal muscle tone, and propulsive activity began. (*b*) In the ileum atropine abolished propulsive activity but not pendular movement.

In concentrations of $10^{-6}$–$10^{-5}$ it completely abolished peristalsis but left the pendular movements unaffected (Fig. 4*b*).

In the guinea-pig atropine $10^{-5}$ abolished the peristaltic reflex of the small and the large intestine alike.

### *Effects of hexamethonium and nicotine*

Hexamethonium in concentrations up to $1 \times 10^{-3}$ inhibited propulsive activity in the rabbit colon only temporarily; after 5–10 min propulsive waves reappeared while hexamethonium was still present in the bath. However, one-tenth of this hexamethonium concentration, i.e. $1 \times 10^{-4}$, abolished all contractions evoked by extrinsic nerve stimulation.

Apart from its initial stimulating effect, nicotine acted like hexamethonium, and concentrations up to $10^{-4}$ failed to abolish propulsive contractions in the rabbit colon, whereas $3 \times 10^{-6}$ was sufficient to abolish the response to pelvic nerve stimulation. It is for this reason that the propulsive activity in the rabbit colon has not been called peristalsis throughout this paper.

In the small intestine of the rabbit both hexamethonium and nicotine blocked peristalsis, as they did also in the guinea-pig small and large intestine.

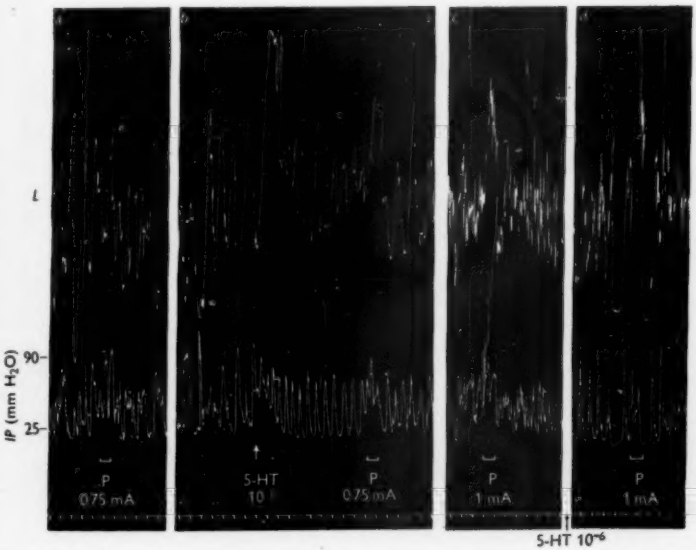


Fig. 5. Rabbit colon. Effect of serosal application of 5-HT. (*a*) Subthreshold pelvic nerve stimulation at P (0·75 mA) before 5-HT. (*b*) Effect of 5-HT $10^{-6}$, applied serosally, on propulsive activity and on pelvic nerve stimulation; note that stimulus (0·75 mA) previously subthreshold became effective. (*c*) Slightly stronger stimulus at P (1 mA), before 5-HT, and (*d*) repeated after 5-HT $10^{-6}$.

### *The effect of serosal application of 5-HT on propulsive activity and on pelvic nerve stimulation*

When 5-HT $10^{-6}$–$10^{-5}$ was applied to the serosal surface of the colon of rabbits and guinea-pigs by addition to the bath its first effect was an immediate contraction of the longitudinal muscle lasting about 1 min. In the rabbit colon this was followed by stimulation of propulsive activity (Fig. 5*b*). In the guinea-pig colon peristalsis was abolished within 2–3 min, but irregular propulsive waves reappeared spontaneously after about 10–20 min while the 5-HT was still present in the bath.

The response to pelvic nerve stimulation was facilitated by 5-HT. Figure 5 shows a typical experiment on rabbit colon. In the presence of 5-HT ($10^{-6}$) a subthreshold stimulus (0·75 mA) became effective and a slightly stronger stimulus (1 mA) caused a much greater response than before. In guinea-pig colon, despite the inhibition of the peristaltic reflex by externally applied 5-HT, the circular contraction in response to pelvic nerve stimulation was nevertheless potentiated, but the contraction was not sustained.

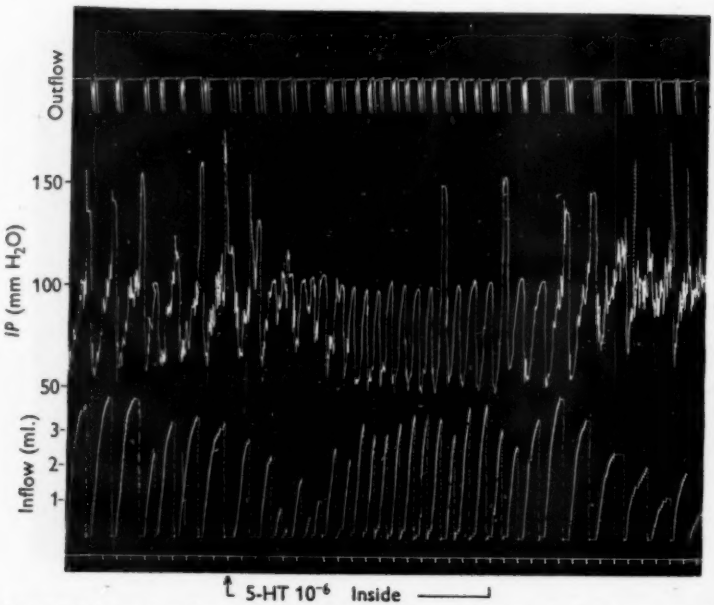


Fig. 6. Guinea-pig colon. Effect of intraluminal application of 5-HT on peristaltic events. From above downwards: outflow (1 ml. per line), intraluminal pressure at oral end, and inflow (ml.) per peristaltic wave. 5-HT at first diminished the inflow by causing circular muscle contraction, but later increased fluid propulsion.

*The effect of intraluminal application of 5-HT on peristalsis*

In guinea-pig colon intraluminal application of 5-HT $10^{-8}$–$10^{-6}$ stimulated peristalsis. In sensitive preparations its first effect was a contracture of the circular muscle which prevented fluid from entering the lumen. The decrease of inflow due to incomplete relaxation is shown in Fig. 6. Next there followed a period of increased peristaltic activity. The threshold fell from 75–100 mm to about 60 mm water. Irregular, non-propulsive contractions were abolished and the peristaltic waves became more frequent and more co-ordinated. As the rapid ejection of fluid allowed a rapid

filling, the fluid transport was almost doubled. When 5-HT was washed out there was usually some inhibition. If 5-HT $10^{-6}$ was given intraluminally over longer periods, or if higher concentrations were used, the initial stimulation gave way to inhibition while 5-HT was still present.

In rabbit colon the effect of 5-HT applied to the mucosa was less readily obtainable but qualitatively not different from that found in guinea-pigs.

### *The release of 5-HT*

*The release of 5-HT into the fluid passing through the lumen of the colon.* Samples of fluid expelled from the ascending colons of guinea-pigs, and from the descending colons of guinea-pigs and rabbits, were collected and

TABLE 1. Release of 5-HT and fluid transport by isolated intestine

| Preparation used | Filling pressure (mm $H_2O$) | 5-HT base release (ng/min ± S.E.) | Fluid transport (ml./min) | No. of expts. | Author |
|---|---|---|---|---|---|
| Guinea-pig, ileum* | 17–20 | 5·74 ± 0·818 | 2·15 | 13 | Bülbring & Lin |
| Guinea-pig, ileum* | 25–30 | 9·59 ± 1·779 | 3·36 | 7 | Bülbring & Lin |
| Rabbit, jejunum* | 25–30 | 9·14 ± 1·905 | 4·03 | 3 | Bülbring & Lin |
| Guinea-pig, oral part of distal colon | 100–110 | 6·33 ± 1·202 | 4·07 | 6 | This paper |
| Guinea-pig, caudal part of distal colon | 100–110 | 1·57 ± 0·354 | 4·11 | 6 | This paper |
| Rabbit, caudal part of distal colon | 80–170 | 0·89 ± 0·204 | 4·40 | 10 | This paper |

* Calculated from Table 1, *J. Physiol.* **140**, 390.

the 5-HT content was determined. In experiments on the small intestine, Bülbring & Lin (1958) found that it was possible to adjust the volume of fluid passing through the intestinal lumen during periods of inactivity to be comparable with the volume which was propelled during active peristalsis. In the oral part of the distal colon, however, because of the high tone of its circular muscle, this was not possible unless the muscular activity was abolished either by adrenaline or by asphyxiation of the muscular layers. While the amounts of 5-HT released from the oral part of the distal colon were of the same order of magnitude as those found by Bülbring & Lin (1958) in the small intestine (Table 1), the amounts from the caudal part were much smaller. This difference was found to be highly significant ($P = 0{\cdot}01$). The difference between the amounts of 5-HT released in guinea-pigs and those in rabbits was not significant.

In four experiments the effect of the intraluminal pressure on the 5-HT release was tested by raising the filling pressure from subthreshold (10–30 mm) to above the threshold at which peristalsis occurred (100–110 mm $H_2O$). Table 2 shows that the 5-HT release was increased from a mean of

1·79 ng/min to 7·57 ng/min. However, no increase of 5-HT release could be found by raising the intraluminal pressure if the peristaltic contractions were completely abolished either by adding adrenaline to the bath, attaining a concentration of $5 \times 10^{-7}$, or by asphyxiation of the muscular layers through stopping the oxygenation of the Tyrode's solution. These experiments indicated that the amounts of 5-HT released into the lumen were directly related to the peristaltic activity.

*The effect of extrinsic nerve stimulation on 5-HT release.* The following experiments were carried out on isolated rabbit colon. The fluid expelled during 3 min periods was collected and the 5-HT content was estimated. The percentage changes of 5-HT release in two successive samples ranged

TABLE 2. 5-HT release in relation to peristalsis in isolated guinea-pig colon

| Experimental conditions and no. of expt. | Below threshold pressure (10–30 mm $H_2O$) | | Above threshold pressure (100–110 mm $H_2O$) | |
|---|---|---|---|---|
| | 5-HT release (ng/min) | Fluid passive flow (ml./min) | 5-HT release (ng/min) | Fluid transport (ml./min) |
| Normal | | | | |
| 1 | 1·40 | 0·35 | 9·00 | 1·8 |
| 2 | 0·66 | 0·82 | 4·48 | 5·6 |
| 3 | 4·37 | 2·75 | 7·50 | 5·0 |
| 4 | 0·75 | 0·25 | 9·30 | 6·2 |
| Mean | 1·79 | 1·04 | 7·57 | 4·65 |
| Adrenaline | | | | |
| 5 | 0·34 | 2·13 | 0·40 | 3·55 |
| Asphyxia | | | | |
| 6 | 1·12 | 3·90 | 0·54 | 1·80 |
| 7 | 4·67 | 2·16 | 4·78 | 2·40 |
| 8 | 2·97 | 0·64 | 3·11 | 1·53 |
| Mean | 2·28 | 2·21 | 2·21 | 2·32 |

from $-40{\cdot}7\,\%$ to $+32{\cdot}3\,\%$ (17 observations) with a mean value of $-3{\cdot}28\,\%$ (S.E. $\pm 4{\cdot}56$). Some of these changes were due to unavoidable sampling errors, since the numbers of peristaltic waves during the sampling periods were not always the same. In general, however, the amount of 5-HT found in successive samples declined slightly with time. The lumbar sympathetic outflow or the pelvic nerve were stimulated for 1 min periods at a frequency of 10/sec. The mean of the amounts of 5-HT found in the control samples collected before nerve stimulation was taken as 100%, and the content of each sample during and after nerve stimulation was calculated as a percentage thereof. The percentage changes in 5-HT release after pelvic nerve stimulation (nine experiments) ranged from $+31{\cdot}7\,\%$ to $-18{\cdot}2\,\%$ with a mean value of $+2{\cdot}25\,\%$ (S.E. $\pm 4{\cdot}65$), which was not significantly different from that of the control samples. Sympathetic stimulation (nine experiments) reduced the amount of 5-HT released

by 29·6% (S.E. ±6·76), which was significantly different from that of the control samples ($P = 0·01$). This reduction appeared to be due to the inhibition of peristaltic activity by sympathetic stimulation; however, it is small compared with the changes produced by altering the filling pressure.

## DISCUSSION

Bayliss & Starling (1900) noted that in the dog and in the rabbit the behaviour of the large intestine differed from that of the small intestine in that the peristaltic contractions were larger, less frequent and more prolonged. Straub & Schild (1933) made similar observations in the cat and guinea-pig. They stated that the main characteristic of the function of the large intestine was the intense sluggishness of all processes and the pronounced tonic quality of the contractions. These observations were confirmed and extended by the present study of the behaviour of isolated loops of large intestine. The finding that the oral part of the distal colon is much more tonic and shows a greater non-propulsive activity than the caudal part may have some physiological significance. In this connexion it is interesting to note that the amount of 5-HT released from the oral part is much greater than that from the caudal part. The observation, that *in situ* (Straub & Schild, 1933) the lumen must be distended before peristaltic waves can occur, was confirmed *in vitro*.

While sympathetic stimulation inhibits peristalsis by relaxation of both muscle coats, parasympathetic stimulation does so by causing a maintained contraction which prevents the distension of the lumen and thereby inhibits peristalsis. Although parasympathetic stimulation can produce an immediate peristaltic wave, it does not increase the frequency of contractions. The finding of Garry & Gillespie (1955) that stimulation of the lumbar sympathetic outflow always causes inhibition of the longitudinal contractions was confirmed. However, it was also found that sympathetic stimulation frequently caused circular contractions at the oral portion of the colon, which were completely abolished by atropine as well as by hexamethonium and nicotine. These observations indicate that the lumbar colonic nerves contain some preganglionic parasympathetic fibres, as suggested by Varagić (1956) and by Gillespie & Mackenna (1959).

It is interesting to note that even in high concentrations atropine, hexamethonium, or nicotine did not abolish the propulsive activity of the rabbit colon. A possible explanation presents itself at once in the fact that the thickness of the tissue, through which the drugs had to diffuse in order to reach their site of action, was greater in the colon than in the other preparations. However, a difficulty of access is not likely to be the explanation, because the muscle contraction in response to pelvic stimulation was completely abolished by much lower concentrations of

these drugs. In contrast, the peristaltic reflex of the guinea-pig colon, the guinea-pig ileum (Bülbring & Crema, 1958) and also of the rabbit ileum could be abolished by atropine as well as by hexamethonium and nicotine. This indicates that there is an important difference in the mechanism of the peristaltic reflex of the large intestine not only between the rabbit and the guinea-pig, but also between the large and small intestine of the same species.

The effect of 5-HT on the large intestine differed in the two species in the same way as Bülbring & Lin (1958) found it in the small intestine. In guinea-pig colon, if applied to the mucosal surface, 5-HT stimulated the peristaltic reflex; this was followed by depression. If applied to the serosal surface, 5-HT inhibited peristalsis after an initial contraction of the longitudinal muscle. On the other hand, when applied to the serosal surface of rabbit colon, 5-HT stimulated propulsive activity after an initial contraction, and inhibition was never observed. When 5-HT was applied to the mucosa of rabbit colon, the stimulating effect was less readily obtainable than in guinea-pigs.

In the rabbit colon 5-HT not only facilitated propulsive activity but also the muscle response to pelvic stimulation. This is consistent with the observations of Bülbring & Crema (1958) and Draskoci & Haranath (1959) that 5-HT sensitizes smooth muscle to acetylcholine. Also in the guinea-pig colon, where 5-HT inhibits peristalsis, it nevertheless potentiated the response to pelvic nerve stimulation. The inhibition of the peristaltic reflex in guinea-pig ileum by 5-HT has been attributed to a ganglionic blocking action (Bülbring & Crema, 1958). If so, the finding that serosal application of 5-HT abolished the peristaltic reflex but not the response to pelvic nerve stimulation indicates that the synaptic pathways differ. It may be that the ganglion cells of the enteric reflex arc are different from those on which the extrinsic nerves impinge, a possibility postulated by previous authors in view of the lack of proportionality between the number of enteric neurones (ganglion cells) and that of extrinsic nerve fibres (see Ambache, 1955).

Finally, the amounts of 5-HT released into the lumen during peristalsis of the oral part of the distal colon were found to be the same order of magnitude as those in the small intestine (Bülbring & Lin, 1958), whereas the amounts released from the caudal part of the colon were much smaller. While 5-HT release could be increased about four times by raising the filling pressure from below to above threshold for peristaltic activity, extrinsic nerve stimulation produced relatively small changes which were directly related to the effect produced on peristaltic activity. Therefore it is concluded that the 5-HT release is controlled by an intrinsic mechanism connected with peristalsis and is not controlled by the extrinsic nerves.

SUMMARY

1. Propulsive activity was recorded in isolated guinea-pig and rabbit colon preparations and the effect of extrinsic nerve stimulation on propulsive activity and on the release of 5-hydroxytryptamine (5-HT) was studied.

2. The threshold pressure required to elicit the peristaltic reflex was higher than for the small intestine. A distension of the lumen was essential for eliciting the peristaltic reflex. The peristaltic wave was much slower and more prolonged than that of the small intestine.

3. Sympathetic stimulation inhibited peristalsis and invariably caused immediate relaxation of the longitudinal muscle, but the effect on the circular muscle was not constant. In those experiments in which sympathetic stimulation caused circular muscle contractions these were completely abolished by atropine, hexamethonium or nicotine.

4. Pelvic nerve stimulation caused a sustained contraction of both muscle coats and during this contraction fluid propulsion was prevented. Atropine $10^{-5}$, hexamethonium $5 \times 10^{-4}$, and nicotine $3 \times 10^{-6}$ abolished the response to pelvic nerve stimulation.

5. Higher concentrations of atropine ($10^{-4}$), hexamethonium ($10^{-3}$) and nicotine ($10^{-4}$) failed to abolish propulsive activity in rabbit colon preparations. However, in the rabbit ileum and in the guinea-pig colon and ileum peristalsis was abolished by these drugs.

6. In the guinea-pig colon, as in the small intestine, 5-HT depressed peristalsis after serosal application, but stimulated peristalsis after mucosal application. During irregular muscular activity, 5-HT applied intraluminally abolished non-propulsive contractions and produced co-ordinated waves; higher concentrations caused an initial contraction and stimulation of peristalsis which was followed by depression.

7. In the rabbit colon 5-HT, applied either outside or inside, stimulated propulsive activity after an initial contraction, although the stimulant effect was less readily obtainable if 5-HT was applied inside.

8. The response to pelvic nerve stimulation was facilitated by serosal application of 5-HT in both species.

9. The amounts of 5-HT released into the lumen during peristalsis of the oral portion of guinea-pig distal colon were of the same order of magnitude as in the small intestine, whereas the amounts released from the caudal portion were much smaller in both species.

10. While 5-HT release was increased about four times by raising the filling pressure from below to above threshold for peristalsis in normal conditions, no such increase could be found if the peristaltic contractions were abolished either by adrenaline or by asphyxiation of the muscular layers.

11. Sympathetic nerve stimulation reduced the amount of 5-HT released on the average only by 30%; pelvic nerve stimulation caused no significant change.

12. It is concluded that, in the large intestine, 5-HT has a similar effect on peristalsis as in the small intestine, and that the 5-HT release is controlled by an intrinsic mechanism associated with peristaltic activity but is not controlled by the extrinsic nerves.

I wish to express my cordial thanks to Professor J. H. Burn for the opportunity of working in his department and for his hospitality and to Dr Edith Bülbring for her constant advice and encouragement throughout this work. I wish to record my gratitude to Mr O. B. Saxby for the construction of apparatus. This work was carried out during the tenure of a scholarship awarded by the Sino-British Fellowship Trust for which I wish to express my thanks.

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# THE EFFECT OF HORMONES ON THE PHYSICAL PROPERTIES AND COLLAGEN CONTENT OF THE RAT'S UTERINE CERVIX

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(*Received* 18 *November* 1959)

In a previous paper (Harkness & Harkness, 1959*b*) changes in the physical properties of the rat's cervix in pregnancy were described. These consisted first of a reorganization of the tissues of the walls of the cervical canals to give a larger natural circumference to the connective tissue framework, and secondly of a change in the properties of this framework which made it behave, not as normally like a continuous network, but as one connected by viscous links which slip slowly under comparatively low tensions.

It seemed of interest to determine whether these changes could be produced by hormonal treatment; and the effects of oestradiol, progesterone and relaxin individually, and in combination, on the cervix in spayed rats have now been investigated. It has been found that an increase in the circumference and weight of the cervix up to about the values found on the 17–18th day of pregnancy, together with a decrease in concentration of collagen similar to that found in pregnancy, can be produced by relaxin in combination with oestradiol and progesterone. Some increase in extensibility under prolonged loading is also produced. A preliminary account of this work has already been published (Cullen & Harkness, 1958).

## METHODS

*Animals.* The rats used were albinos of the local strain, weighing 150–180 g and about 3 months old at the time of spaying. Ovaries were removed aseptically by the dorsal route under ether anaesthesia, and administration of hormones began about 3 weeks later.

*Treatment.* The following substances, all given subcutaneously, were used:

(*a*) Oestradiol cyclopentyl-propionate (Depo-Estradiol; Upjohn) in arachis oil.

(*b*) Progesterone, either dissolved in ethyl oleate (Progestin; Organon Laboratories) or progesterone B.P. in olive oil, any which would not dissolve being suspended by homogenization in a Potter–Elvehjem homogenizer.

(*c*) Relaxin powder in 5% (w/w) beeswax in peanut oil (Kroc, Steinetz & Beach, 1959). The preparation was 'Releasin' (Warner–Chilcott): (*A*) Batch no. W 1164 A, lot 53; (*B*) Batch W 1164 A, lot 66; (*C*) Batch W 1164 A, lot 43.

All these preparations have been assayed on the mouse and some on the guinea-pig

against Warner-Chilcott Relaxin Reference Standard W1164A lot 8 (*Std*), of activity 150 guinea-pig units/mg. The figures with 95 % confidence limits (provided by Dr B. G. Steinetz) are given in Table 1.

TABLE 1

| | Mouse assay | Guinea-pig assay |
|---|---|---|
| (*A*) Lot 53 | 0·2 mg *Std*/mg (79–127 %) | — |
| (*B*) Lot 66 | 0·99 mg *Std*/mg (72–140 %) | 1·06 mg *Std*/mg (76–131 %) |
| | 1·06 mg *Std*/mg (77–130 %) | — |
| (*C*) Lot 43 | 0·11 mg *Std*/mg (51–195 %) | 0·21 mg *Std*/mg (64–157 %) |
| | 0·12 mg *Std*/mg (71–141 %) | 0·26 mg *Std*/mg (68–147 %) |
| | 0·13 mg *Std*/mg (81–124 %) | — |

We have recorded dosage in weight of the reference standard by mouse assay, e.g. for (*A*) 1 mg = 0·2 mg of reference standard. Thus, a dose of 1 mg on this scale is equivalent to 150 guinea-pig units.

*Mechanical tests on cervix.* The effect of tension on the walls of the cervix was examined by the same method as that used previously on rats at different times of pregnancy (Harkness & Harkness, 1959*b*), i.e. the excised cervix was suspended in oxygenated Ringer–Locke solution and stretched between two parallel steel rods 0·6 mm in diameter, one through each canal. Some experiments were done at 22° C, some at 37° C. Two types of test were made. *a.* The force pulling the rods apart was increased stepwise by 25 g every 15 sec until the tissues ruptured (22° C).

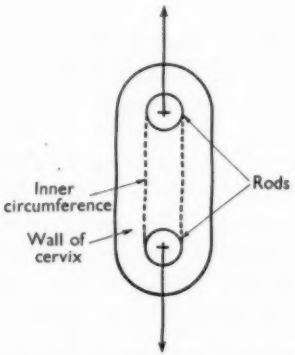


Fig. 1. Diagram of transverse section of cervix under tension. The bar of tissue which separates the two cervical canals and runs between the rods is not represented in the figure.

*b.* The force pulling the rods apart was kept constant for a time and subsequently increased as in (*a*) until the tissues ruptured (37° C).

As before (Harkness & Harkness, 1959*b*) the measurement used to describe the effect of tension is the distance round and between the rods (Fig. 1) and is referred to as the 'inner circumference'. For calculations of tensions per unit cross-sectional area the tissue was regarded as if it were a straight piece of this length and constant cross-sectional area, with density of 1 for whole tissue and 1·4 for collagen. The use of this length is not precise, as the mean circumference may be as much as 50 % higher than the inner circumference and there is a corresponding over-estimate of the cross-sectional area. The errors, are, however, generally similar in the different groups, and their effect is only to produce small alterations

in the absolute magnitude of the differences between groups. For calculation of breaking stress, the mean was taken between the load which broke the tissue and the previous one which just did not. Tests of the first sort (*a*) give length–tension curves of which a typical example is shown in Fig. 2. The circumference increased rapidly at first but more slowly as the load was increased. Then over a wide range of load the curve became approximately a straight line until a little before the point of rupture when the slope usually increased again. When the load was taken off between each addition, there was immediate diminution in circumference, increasing with load and amounting to 10–15 % just before break. This effect was much the same in most groups as in normal non-pregnant animals (Harkness & Harkness, 1959*b*) and will not be discussed. Only the following parameters of the curves were measured: first the circumference ($l_z$) of the cervix at zero load, obtained by extrapolation back from the straight part of the curve; secondly, the slope ($k$) of the linear part of the curve of length against tension; thirdly, the conditions at breaking point.

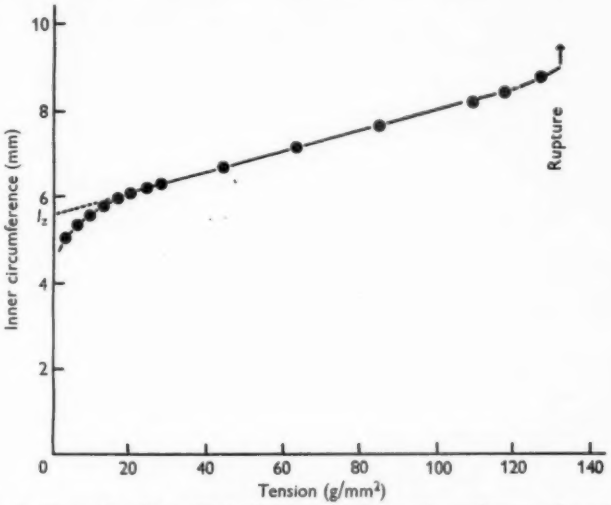


Fig. 2. Example of the effect of tension on inner circumference of cervix.

It is clear that in tissues of the same material and shape the constant $k$ is related to size of the sample, i.e. to $l_z$, and in order to remove this effect we have recorded $k/l_z$ rather than $k$ itself. It is also clear that other things being the same, the value of $k$ is related inversely to the amount of material present which resists stress. Even if one does not know the nature of this material, a measure of it may be obtained from the breaking stress. For measurement of $k$, therefore, tension has been expressed in units of breaking tension ( = 1). The value of $k/l_z$ in these units is the fractional change in length between zero and breaking tension.

The results of these tests have generally shown variations of $k/l_z$ between different treatment groups which are small and inconsistent compared to differences in $l_z$. The main use of these tests is therefore to give a measure of the size (circumference, $l_z$) of the connective-tissue framework of the cervix after different treatments. The comparison between these is hardly affected if the recording is still further simplified by taking only the single measurement of circumference at half breaking tension, as was done in the preliminary communication (Cullen & Harkness, 1958).

Smooth muscle does not appear to play any important part in these tests (Harkness & Harkness, 1959*b*).

In tests of the second sort (*b*), when a constant load is applied for a long time, it has previously been found (Harkness & Harkness, 1959*b*) that the cervix on the last day of pregnancy, after an initial rapid extension, shows a slower prolonged increase in length at constant rate. This rate was measured by the ratio of the slope ($K$) of the linear part of the curve to the length at zero time ($l_0$) obtained by extrapolation (Fig. 3) and the same procedure is used here. $K$ is measured in mm/min. This type of test gives information on the properties of a component of the tissues which behaves as a series viscous element. It is probable that this is an interfibrillary cementing material, and the prolonged extension takes place by the fibrils slipping past each other (Harkness & Harkness, 1959*a*). The rate of this slow extension increases with temperature and the experiments were therefore performed at 37° C to make the measurement easier.

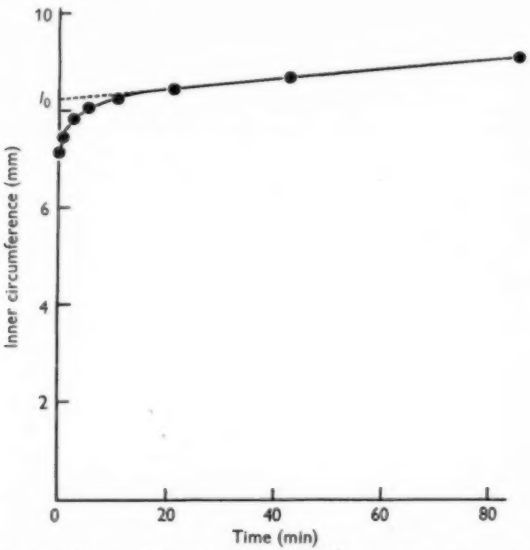


Fig. 3. Example of the effect of prolonged loading on cervix. The cervix, from a rat previously treated with oestradiol, progesterone and relaxin (doses, Table 3), was loaded with 100 g weight.

*Chemical methods.* Hydroxyproline was estimated by the method of Neuman & Logan (1950) on acid hydrolysates (5 ml. 6N-HCl, 4 hr, 40 lb./sq.in. or 2·8 kg/cm² in autoclave) of the whole tissue removed from the apparatus at the end of the experiment. The collagen content of the tissue was obtained by multiplying the hydroxyproline value by 7·46. A number of blanks were done, in which hydrogen peroxide was omitted, to test for the presence in these crude hydrolysates of chromogens, other than hydroxyproline, which might react with the *p*-dimethylamino-benzaldehyde in the last stage of the estimation. No appreciable amounts were found.

## RESULTS

We began by investigating the time course of change in the cervix after administration of oestradiol and progesterone individually and of relaxin in combination with oestradiol. Relaxin was not used alone at this stage

because it is known to have little effect by itself on the symphysis pubis. In these experiments only tests of the first sort (*a*), in which load was increased progressively, were used. On the basis of these experiments others using the same type of test were done with combinations of hormones given for a standard time. On the results of these experiments others were done, again with combinations of hormones given for a standard time, but the cervix was tested by the second method (*b*), with a single load applied for a long time.

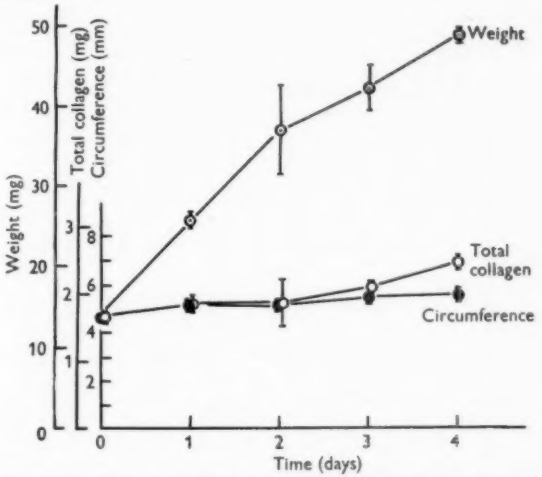


Fig. 4. Effect of oestradiol on weight, total collagen content and inner circumference of cervix. 5 $\mu$g of oestradiol cyclopentyl-propionate was given on day 0. The circumference is that at half breaking tension. The length of the vertical line through each point is twice the standard error of the mean.

### *Time course of change under treatment*

*Oestradiol.* A single dose of oestradiol (5 $\mu$g) produced a rapid increase in weight of tissue and, like oestrone (Harkness, Harkness & Moralee, 1957), a slow increase in the total collagen content of the cervix (Table 2). There was a rise in $k/l_z$ and in the circumference at breaking point. In later experiments the main effect of oestradiol alone was on $l_z$. The principal point of interest shown by these preliminary experiments was that the total effect on circumference was small, being more like that on collagen content than on weight. The relations between the changes in weight and collagen content, and the results of the mechanical tests summarized by taking circumference at half the breaking tension, are shown in Fig. 4. Breaking stress per unit cross-sectional area of tissue decreased to about half, but this could be accounted for simply by the fall in concentration of collagen resulting from its slow growth compared with the increase in

TABLE 2. Effect of oestradiol and progesterone on cervix of spayed rat

| Treatment | Body wt. (g) | Wt. of cervix (mg) | Total collagen (mg) | Concn. collagen (g/100 g) | $l_z$ (mm) | $k/l_z$ | Circumference at break (mm) | Stress at break (g/mm²) | Stress at break (kg/mm² coll.) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Oestradiol (5 μg) | | | | | |
| Untreated | 228 ± 5 | 13·9 ± 0·8 | 1·67 ± 0·09 | 12·0 ± 0·5 | 4·02 ± 0·16 | 0·36 ± 0·02 | 6·07 ± 0·21 | 91 ± 3 | 1·07 ± 0·03 |
| 1 day | 203 ± 12 | 26·0 ± 1·0 | 1·85 ± 0·14 | 7·1 ± 0·6 | 3·96 ± 0·09 | 0·52 ± 0·04 | 6·50 ± 0·17 | 73 ± 9 | 1·44 ± 0·13 |
| 2 days | 211 ± 6 | 37·2 ± 5·5 | 1·87 ± 0·37 | 6·7 ± 0·7 | 3·87 ± 0·10 | 0·63 ± 0·09 | 6·63 ± 0·48 | 54 ± 5 | 1·55 ± 0·24 |
| 3 days | 205 ± 13 | 42·3 ± 3·0 | 2·11 ± 0·08 | 6·8 ± 0·6 | 4·26 ± 0·14 | 0·46 ± 0·12 | 7·25 ± 0·67 | 52 ± 3 | 2·14 ± 0·07 |
| 4 days | 210 ± 2 | 47·6 ± 0·8 | 2·48 ± 0·11 | 7·0 ± 0·3 | 4·22 ± 0·20 | 0·60 ± 0·04 | 7·35 ± 0·20 | 40 ± 4 | 1·07 ± 0·14 |
| | | | | Progesterone (10 mg/day) | | | | | |
| *A.* Untreated | 206 ± 15 | 15·6 ± 1·2 | 1·84 ± 0·09 | 11·7 ± 0·4 | 3·45 ± 0·17 | 0·40 ± 0·02 | 5·05 ± 0·25 | 92 ± 6 | 1·07 ± 0·04 |
| 1 day | 212 ± 11 | 16·1 ± 0·5 | 1·84 ± 0·06 | 11·4 ± 0·5 | 3·62 ± 0·30 | 0·42 ± 0·02 | 4·98 ± 0·47 | 101 ± 2 | 1·25 ± 0·27 |
| 2 days | 217 ± 11 | 21·1 ± 2·1 | 2·44 ± 0·31 | 11·5 ± 0·6 | 3·58 ± 0·11 | 0·44 ± 0·04 | 5·15 ± 0·19 | 151 ± 8 | 1·22 ± 0·10 |
| 3 days | 207 ± 5 | 19·1 ± 1·5 | 2·26 ± 0·20 | 12·1 ± 1·3 | 3·42 ± 0·11 | 0·38 ± 0·02 | 6·70 ± 0·58 | 99 ± 12 | 1·13 ± 0·03 |
| 9 days | 219 ± 9 | 16·7 ± 2·5 | 2·14 ± 0·13 | 13·1 ± 1·4 | 3·30 ± 0·06 | 0·51 ± 0·13 | 5·22 ± 0·37 | 114 ± 19 | 1·25 ± 0·22 |
| *B.* Untreated | 264 ± 8 | 18·5 ± 1·8 | 2·08 ± 0·23 | 10·7 ± 0·2 | 3·15 ± 0·15 | 0·28 ± 0·06 | 4·97 ± 0·12 | 86 ± 4 | 1·12 ± 0·07 |
| 9 days | 264 ± 14 | 53·5 ± 6·0 | 2·90 ± 0·34 | 8·5 ± 0·1 | 4·84 ± 0·14 | 0·39 ± 0·06 | 7·91 ± 0·55 | 43 ± 6 | 1·09 ± 0·15 |

There were three rats in each group. Treatment with oestradiol was with 5 μg cyclopentyl-propionate in 0·1 ml. arachis oil on day 0, progesterone 10 mg/day in 0·5 ml. olive oil. The meaning of $l_z$ and $k$ are given in text. The estimate of variation is the standard error of the mean.

weight of the whole tissue. When breaking stress was expressed in terms of collagen, in this, as in subsequent experiments, no evidence of change was found (Table 2).

*Progesterone.* A dose of 10 mg/day was used, as this is about the minimum required to maintain pregnancy after spaying (Alexander & Frazer, 1954). In the first experiment (Table 2, *A*) a commercial preparation (Progestin), in which 10 mg were dissolved in 0·4 ml. of ethyl-oleate, had no effect. In a second experiment (Table 2, *B*) progesterone homogenized

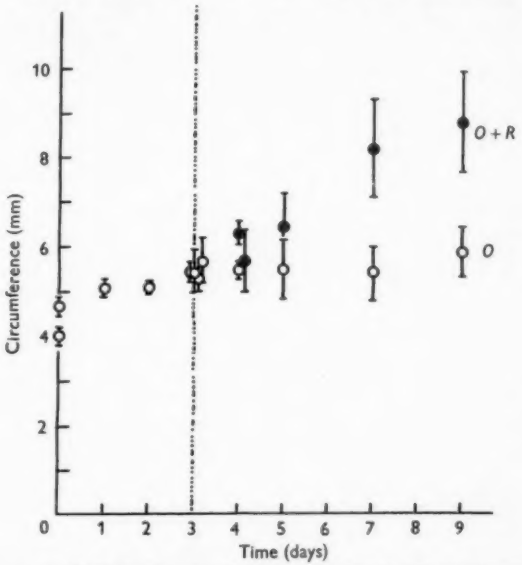


Fig. 5. Summary of effect of oestradiol (*O*) and relaxin (*R*) on circumference of cervix. This figure summarizes all results for animals treated with oestradiol (*O*), with and without relaxin (*R*) (Tables 1, 2 and 3). The circumference is that at half breaking tension. The estimate of variation shown by the vertical lines is the standard error of the mean. Treatment with oestradiol began on day 0, with relaxin on day 3.

in olive oil, partly in solution and partly in suspension, produced a small but significant increase in weight of cervix and in $l_z$.

*Relaxin.* In the first experiment the animals were previously treated with oestradiol and 3 days later given a single dose of 0·5 mg of relaxin (*A*). They were examined 24 hr later and no significant effect was found (Table 3, *A*). The experiment was repeated with twice and four times the original dose of relaxin, again without significant effect (Table 3, *B*); but when administration of relaxin was prolonged (4 days) a significant increase in $l_z$ was produced (Table 3, *C*; Fig. 5).

TABLE 3. The effect of relaxin on the cervix in spayed rats previously treated with oestradiol

| Dose of relaxin (mg *Std*)* | Day killed | Body wt. (g) | Wt. of cervix (mg) | Total collagen (mg) | Concn. collagen (g/100 g) | $l_z$ (mm) | $k/l_z$ | Circumference at break (mm) | Stress at break (kg/mm² coll.) |
|---|---|---|---|---|---|---|---|---|---|
| *A.* Control | 4 | 200 ± 24 | 63 ± 9 | 3·6 ± 1·0 | 6·0 ± 0·3 | 4·33 ± 0·28 | 0·51 ± 0·06 | 7·00 ± 0·43 | 1·01 ± 0·08 |
| Relaxin 0·5 | 4 | 224 ± 16 | 63 ± 10 | 3·5 ± 0·9 | 5·4 ± 0·8 | 4·89 ± 0·35 | 0·59 ± 0·11 | 8·42 ± 0·60 | 1·04 ± 0·16 |
| *B.* Control | 4 | 227 ± 17 | 63 ± 16 | 4·4 ± 1·1 | 6·9 ± 0·1 | 4·43 ± 0·40 | 0·39 ± 0·09 | 6·95 ± 0·35 | 1·01 ± 0·28 |
| Relaxin 0·5 | 4 | 231 ± 10 | 71 ± 16 | 4·7 ± 1·2 | 6·6 ± 0·3 | 4·57 ± 0·37 | 0·43 ± 0·12 | 7·82 ± 1·42 | 1·04 ± 0·09 |
| Relaxin 1·0 | 4 | 266 ± 10 | 48 ± 7 | 3·0 ± 0·3 | 6·3 ± 0·6 | 4·97 ± 0·47 | 0·32 ± 0·06 | 6·90 ± 0·42 | 0·86 ± 0·06 |
| Relaxin 2·0 | 4 | 214 ± 48 | 71 ± 20 | 5·1 ± 1·0 | 6·9 ± 0·5 | 4·92 ± 0·23 | 0·36 ± 0·08 | 7·39 ± 1·09 | 0·85 ± 0·12 |
| *C.* Control | 3 | 246 ± 9 | 78 ± 11 | 7·4 ± 1·9 | 9·2 ± 1·3 | 4·65 ± 0·55 | 0·37 ± 0·05 | 6·40 ± 0·67 | 0·62 ± 0·10 |
| Control | 5 | 261 ± 6 | 74 ± 5 | 4·2 ± 0·5 | 5·7 ± 0·4 | 4·20 ± 0·06 | 0·58 ± 0·11 | 7·95 ± 0·77 | 0·82 ± 0·06 |
| Control | 7 | 285 ± 6 | 59 ± 1 | 3·9 ± 0·4 | 6·6 ± 0·5 | 4·28 ± 0·30 | 0·51 ± 0·03 | 8·04 ± 0·38 | 0·83 ± 0·09 |
| Relaxin 0·5 × 1 | 5 | 293 ± 5 | 60 ± 1 | 3·2 ± 0·4 | 5·4 ± 0·5 | 4·83 ± 0·31 | 0·64 ± 0·08 | 8·31 ± 0·93 | 0·84 ± 0·08 |
| Relaxin 0·5 × 2† | 7 | 271 ± 19 | 94 ± 11 | 5·6 ± 3·0 | 5·5 ± 1·6 | 6·66 ± 0·82 | 0·52 ± 0·14 | 10·75 ± 0·24 | 1·14 ± 0·15 |

There were three rats in each group. The estimate of variation is the standard error of the mean. All animals were given oestradiol cyclopentyl-propionate (5 $\mu$g) on day 0; relaxin (*A*) was given on day 3, or days 3 and 5 (†). **Std*, see Table 1. The meanings of $l_z$ and $k$ are stated in the text.

TABLE 4. The effect of combinations of oestradiol, progesterone and relaxin on the cervix of spayed rats. Experiment 1

| Treatment | 1 Body wt. (g) | 2 Wt. of cervix (mg) | 3 Total collagen (mg) | 4 Concn. collagen (g/100 g) | 5 $l_z$ (mm) | 6 $k/l_z$ | 7 Breaking load (g) | 8 Circumference at break (mm) | 9 Stress at break (g/mm²) | 10 Stress at break (kg/mm² coll.) |
|---|---|---|---|---|---|---|---|---|---|---|
| None | 281 ± 7 | 18·7 ± 2·2 | 2·53 ± 0·12 | 13·8 ± 0·9 | 3·47 ± 0·17 | 0·32 ± 0·02 | 804 ± 148 | 4·95 ± 0·29 | 105 ± 10 | 1·11 ± 0·18 |
| Oestradiol (*O*) | 268 ± 26 | 66·0 ± 14·0 | 4·45 ± 0·96 | 6·7 ± 0·2 | 4·96 ± 0·40 | 0·37 ± 0·05 | 729 ± 36 | 8·40 ± 1·21 | 48 ± 3 | 1·02 ± 0·07 |
| Progesterone (*P*) | 258 ± 3 | 15·3 ± 4·1 | 2·13 ± 0·24 | 15·5 ± 3·0 | 3·55 ± 0·15 | 0·37 ± 0·07 | 587 ± 50 | 5·13 ± 0·31 | 108 ± 18 | 1·01 ± 0·07 |
| Relaxin (*R*) | 248 ± 3 | 14·0 ± 1·5 | 1·28 ± 0·48 | 8·7 ± 1·7 | 3·90 ± 0·21 | 0·32 ± 0·04 | 654 ± 178 | 5·49 ± 0·13 | 127 ± 33 | 2·39 ± 0·96 |
| *PR* | 258 ± 4 | 19·7 ± 2·4 | 2·21 ± 0·40 | 11·0 ± 0·7 | 3·85 ± 0·31 | 0·36 ± 0·10 | 679 ± 54 | 5·45 ± 0·33 | 94 ± 21 | 1·19 ± 0·19 |
| *OP* | 251 ± 5 | 47·0 ± 8·7 | 3·37 ± 0·16 | 7·5 ± 1·0 | 5·30 ± 0·34 | 0·57 ± 0·03 | 662 ± 72 | 8·40 ± 0·73 | 63 ± 13 | 1·05 ± 0·10 |
| *OR* | 238 ± 4 | 84·3 ± 5·7 | 4·03 ± 0·24 | 4·8 ± 0·4 | 7·30 ± 1·00 | 0·44 ± 0·06 | 637 ± 9 | 11·15 ± 0·91 | 43 ± 7 | 1·28 ± 0·18 |
| *OPR* | 255 ± 7 | 97·0 ± 13·0 | 4·70 ± 0·76 | 4·9 ± 0·2 | 8·70 ± 0·59 | 0·61 ± 0·16 | 362 ± 29 | 14·22 ± 0·67 | 27 ± 6 | 0·80 ± 0·17 |

There were three rats in each group. The estimate of variation is the standard error of the mean. *O* = oestradiol cyclopentyl-propionate 5 $\mu$g on day 0. *P* = progesterone 10 mg/day. *R* = relaxin (*A*) 0·5 mg *Std* on days 3, 5 and 7. The meanings of $l_z$ and $k$ are stated in the text.

*Combinations of hormones*

In the next experiment we increased the duration of relaxin treatment by a further 2 days and used all three hormones, oestradiol, relaxin and progesterone, in all possible combinations. The results are shown in Table 4 and an analysis of variance on them in Table 5. Both oestradiol and

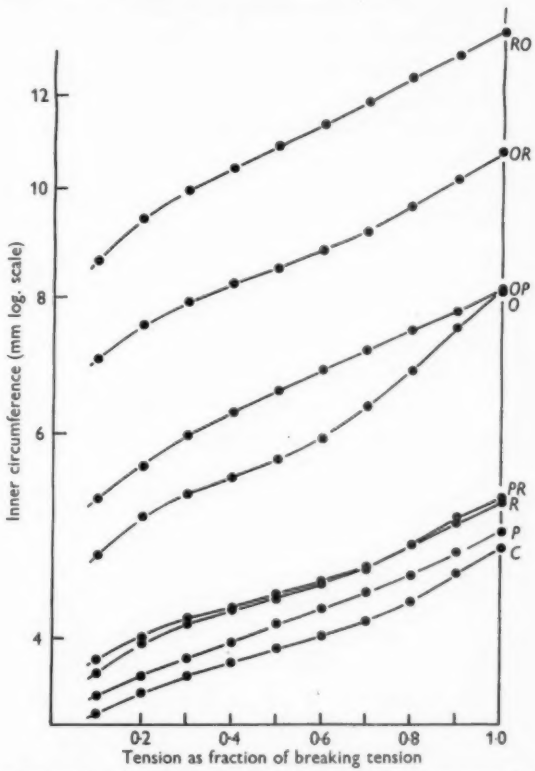


Fig. 6. The effect of tension on circumference of cervix of rats treated in differen ways. Each curve is the mean for all rats in the group. The treatment is given at the end of each curve, $C$ = no treatment, $O$ = oestradiol, $P$ = progesterone, $R$ = relaxin. The doses are given in Table 3.

relaxin produced effects. These were (*a*) an increase in $l_z$ and in circumference at time of rupture (Table 4, cols. 5 and 8), the whole length–tension curve being shifted bodily upward without much change in its shape (Fig. 6); (*b*) an increase in the wet weight of the cervix (Table 4, col. 2); (*c*) a diminution in the concentration of collagen (Table 4, col. 4). There is also a significant interaction in the statistical sense (Table 5) between

$P$ = progesterone 10 mg/day, $R$ = relaxin (*A*) 0·5 mg *Std* on days 3, 5 and 7. The meanings of $l_z$ and *k* are stated in the text.

oestradiol and relaxin, which means that the combined effect was greater than was to be expected from simple addition of the individual effects, relaxin given alone having no significant effect. The largest effect on circumference in this experiment was obtained when progesterone was combined with oestradiol and relaxin. Though the effect of progesterone was not significant statistically at the 5% level, it appears to be a real one as it was found again in later experiments. It will be seen in Table 6 that $l_0$ obtained by extrapolation to zero time with constant load, like $l_z$ in Table 4, is greater in the *OPR* than in the *OR* group. The effects of different combinations of hormones on $l_0$ and $l_z$ are obviously similar (compare Tables 4 and 6) and if the results are combined the difference between the *OR* and the *OPR* groups is significant ($0{\cdot}02 > P > 0{\cdot}01$).

TABLE 5. Significance of results of Experiment 1 with oestradiol, progesterone and relaxin (Table 4)

| | Primary effects | | | Interactions | | | |
|---|---|---|---|---|---|---|---|
| | *O* | *R* | *P* | *PR* | *OP* | *OR* | *OPR* |
| Weight of cervix | 0·001 | 0·01 | — | — | — | 0·01 | — |
| Total collagen | 0·001 | — | — | 0·05 | — | — | — |
| Concn. of collagen | 0·001 | 0·01 | — | — | — | — | — |
| $l_z$ | 0·001 | 0·001 | — | — | — | 0·01 | — |
| $K/l_z$ | 0·05 | — | 0·05 | — | — | — | — |
| Circumference at break (mm) | 0·001 | 0·001 | — | — | — | 0·001 | — |
| Stress at break (g/mm²) | 0·001 | — | — | — | — | — | — |

The figures in the table are values of $P$ in analysis of variance, and are maxima, i.e. $P$ of 0·05 means $0{\cdot}05 > P > 0{\cdot}01$, $P$ of 0·01 means $0{\cdot}01 > P > 0{\cdot}001$.

*Effect of prolonged loading.* The tests used in the previous experiments were conducted at 22° C with rapidly increasing load. They would be expected to bring out any effects of hormones on the dimensions of the cervix, but would not give evidence whether any of them would cause the tissues to show the prolonged slow extension under low load seen at the end of pregnancy (Harkness & Harkness, 1959*b*). We therefore repeated the last experiment with the three hormones in all possible combinations with the tissues in Locke's solution at 37° C, maintaining a constant load for 1½ hr and measuring the rate of increase of the circumference over the last half of the period. In Table 6 this slope ($K$) is recorded as a proportion of the circumference at zero time ($l_0$) obtained by extrapolation. In none of the groups of animals was there any prolonged extension comparable in rate to that seen at the end of pregnancy, nor in a second experiment (Table 7) did increasing the dose of relaxin eight times produce any increase in $K/l_0$, values still being lower than at the end of pregnancy for comparable conditions. Increasing the dose of relaxin did, however, increase the value of $l_0$. In this second experiment only animals treated with oestradiol and

TABLE 6. The effect of combinations of oestradiol, progesterone and relaxin on the cervix of spayed rats. Experiment 2

| Treatment | Body wt. (g) | Wt. of cervix (mg) | Total collagen (mg) | Concn. collagen (g/100 g) | $l_0$ (mm) | $K/l_0$ for 100 g load | Stress* at $l_0$ (g/mm² coll.) | Circumference at break (mm) | Stress at break (g/mm²) | Stress at break (kg/mm² coll.) |
|---|---|---|---|---|---|---|---|---|---|---|
| None | 207 ± 20 | 20·7 ± 1·5 | 1·97 ± 0·40 | 9·6 ± 0·9 | 3·36 ± 0·09 | < 0·1 | 112 ± 9 | 4·82 ± 0·19 | 119 ± 12 | 1·74 ± 0·01 |
| Oestradiol (*O*) | 199 ± 3 | 53·3 ± 2·7 | 3·41 ± 0·59 | 4·6 ± 1·1 | 4·84 ± 0·07 | 0·53 ± 0·09 | 103 ± 20 | 7·90 ± 0·78 | 56 ± 1 | 1·26 ± 0·17 |
| Progesterone (*P*) | 228 ± 33 | 21·8 ± 4·7 | 1·85 ± 0·29 | 9·0 ± 0·6 | 3·25 ± 0·51 | 0·29 ± 0·29 | 124 ± 37 | 4·73 ± 0·95 | 100 ± 15 | 1·55 ± 0·13 |
| Relaxin (*R*) | 214 ± 12 | 25·7 ± 4·0 | 2·36 ± 0·18 | 9·4 ± 0·8 | 3·70 ± 0·04 | < 0·1 | 110 ± 7 | 5·50 ± 0·24 | 90 ± 4 | 1·39 ± 0·10 |
| *PR* | 216 ± 20 | 22·3 ± 3·7 | 1·99 ± 0·08 | 9·3 ± 0·2 | 3·57 ± 0·04 | < 0·1 | 119 ± 3 | 4·79 ± 0·42 | 104 ± 29 | 1·55 ± 0·13 |
| *OP* | 216 ± 8 | 60·0 ± 11·7 | 3·33 ± 0·65 | 5·6 ± 0·1 | 4·28 ± 0·50 | 0·20 ± 0·20 | 92 ± 8 | 7·02 ± 0·71 | 66 ± 5 | 1·67 ± 0·13 |
| *OR* | 234 ± 8 | 96·6 ± 3·6 | 3·55 ± 0·03 | 3·7 ± 0·1 | 7·22 ± 0·30 | 1·27 ± 0·63 | 143 ± 5 | 11·02 ± 0·18 | 43 ± 3 | 1·68 ± 0·08 |
| *OPR* | 225 ± 28 | 122·4 ± 1·4 | 4·55 ± 0·40 | 3·7 ± 0·3 | 10·55 ± 0·35 | 1·24 ± 0·37 | 164 ± 9 | 16·00 ± 1·10 | 37 ± 11 | 1·43 ± 0·43 |

The estimate of variation is the standard error of the mean. There were two rats in each group. The dose of oestradiol was 10 μg cyclopentyl-propionate on day 0; progesterone 10 mg/day from day 3 onwards; relaxin 0·5 mg *Std* on days 3, 5, 7 and 9. The rats were killed on days 10 and 11, the cervices of those killed on day 10 being loaded initially with 50 g, those on day 11 with 100 g. The value of $K/l_0$ for 50 g load was multiplied by 2 to combine with values for 100 g load. The meanings of $l_0$ and $K$ are given in the text. * For 100 g load.

progesterone, with or without relaxin, were used. We have also recorded in Table 7 the value $l_1$ in the equation $l = l_1 + a \log t$, where $l$ is circumference and $t$ is time (1 unit = 5 sec). This describes fairly well the initial effects of loading, and allows the results to be compared with those reported previously for normal and pregnant animals (Harkness & Harkness, 1959*b*). In further experiments in which samples of relaxin of different degrees of purity were used and the dosage taken up to 40 times the original, some further increase in $l_0$ was found, and a small increase in $K/l_0$, but still not to values as high as at the end of pregnancy (Table 8, Fig. 7).

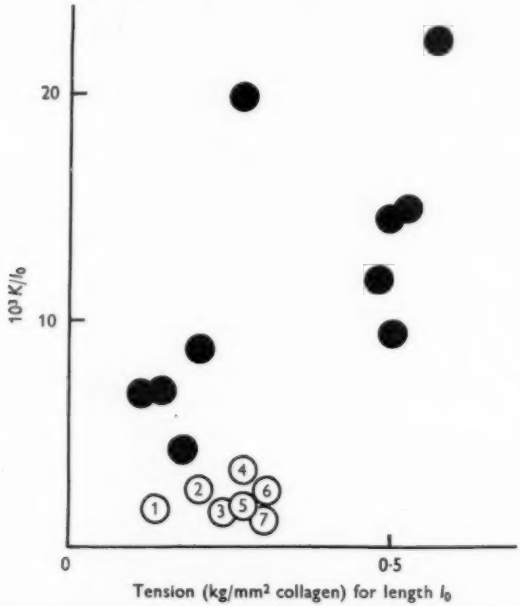


Fig. 7. Relation between rate of extension under prolonged loading (ordinate, $K/l_0$) and tension per unit cross-sectional area of collagen calculated for length $l_0$ (abscissa). ● 21st day of pregnancy (from Harkness & Harkness, 1959*b*) and ○ relaxin-treated, means from Table 7. Dose (mg) and type of relaxin: 1, 0·5 *C*; 2, 1 *C*; 3, 1 *A*; 4, 10 *C*; 5, 0·5 *B*; 6, 0·5 *A*; 7, 1 *B*.

## DISCUSSION

### *Relation of results to investigation of 'dilatability' of cervix*

One method which has been used to investigate the 'dilatability' or softening of the cervix is to introduce a conical probe into the canal, to push it in until resistance is felt and then to record the diameter (de Vaal, 1946). Crelin (1958) has made this method more quantitative as regards the

TABLE 7. The effect of different doses of relaxin on cervix in rats treated with oestradiol and progesterone

TABLE 7. The effect of different doses of relaxin on cervix in rats treated with oestradiol and progesterone

| | Dose of relaxin (mg *Std*/day) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Sample *A* (30 g-p u./mg) | | | | Sample *B* (150 g-p u./mg) | | |
| | 0 | 0·5 | 1·0 | 2·0 | 0·5 | 1·0 | 2·0 |
| Weight of cervix (mg) ... | 61 ± 5 | 129 ± 7 | 164 ± 12 | 141 ± 36 | 113 ± 11 | 148 ± 20 | 146 ± 7 |
| Total collagen (mg) | 3·68 ± 0·13 | 4·60 ± 0·06 | 4·77 ± 0·42 | 3·58 ± 0·30 | 4·53 ± 0·89 | 4·72 ± 0·06 | 4·12 ± 0·48 |
| Concn. of collagen (g/100 g) | 6·12 ± 0·24 | 3·59 ± 0·14 | 2·95 ± 0·47 | 2·67 ± 0·49 | 3·99 ± 0·43 | 3·25 ± 0·48 | 2·81 ± 0·03 |
| $l_1$ (mm) | 4·2 ± 0·8 | 7·6 ± 1·3 | 11·5 ± 0·5 | 10·7 ± 1·9 | 8·3 ± 1·2 | 10·4 ± 0·3 | 11·2 ± 0·6 |
| $l_s$ (mm) | 4·8 ± 0·9 | 9·2 ± 1·8 | 13·9 ± 1·0 | 12·3 ± 2·4 | 9·3 ± 1·3 | 11·9 ± 0·1 | 11·8 ± 0·4 |
| $10^3\ K/l_0$ | 0·42 ± 0·14 | 1·19 ± 0·11 | 0·83 ± 0·27 | 1·01 ± 0·15 | 0·69 ± 0·36 | 0·40 ± 0·05 | 0·67 ± 0·15 |
| Stress at $l_0$ (g/mm² collagen) | 91 ± 14 | 131 ± 24 | 205 ± 32 | 237 ± 27 | 154 ± 50 | 176 ± 3 | 240 ± 17 |
| Stress at break (kg/mm² collagen) | 1·16 ± 0·07 | 1·23 ± 0·07 | 1·75 ± 0·11 | 1·10 ± 0·11 | 1·10 ± 0·13 | 1·44 ± 0·29 | 1·44 ± 0·13 |
| Circumference at break (mm) | 6·4 ± 0·6 | 12·8 ± 1·4 | 17·7 ± 0·5 | 15·7 ± 2·0 | 12·4 ± 0·1 - | 15·4 ± 0·6 | 17·4 ± 1·6 |

The estimate of variation is the standard error of the mean. There were two rats in each group. All animals were given oestradiol cyclopentyl-propionate (10 $\mu$g on day 0), and progesterone (10 mg/day from day 3). Relaxin was given every day from day 3 in concentration 5 mg *Std*/ml. in beeswax and oil. The meanings of $l_1$, $l_s$, $l_0$ and $K$ are given in the text. Load 100 g. g-p u. = guinea-pig unit.

TABLE 8. Effect of samples of relaxin of different purity

| | | Dose of relaxin (mg *Std*/day) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Sample *B* (150 g-p u./mg) | | Sample *A* (30 g-p u./mg) | | Sample *C* (18 g-p u./mg) | | |
| | Control 0 | 0·5 | 1·0 | 0·5 | 1·0 | 0·5 | 1·0 | 10·0 |
| Wt of cervix (mg) | 62 ± 1 | 90 ± 4 | 104 ± 5 | 92 ± 3 | 159 ± 3 | 110 ± 1 | 121 ± 14 | 142 ± 12 |
| Total collagen (mg) | 3·49 ± 0·01 | 3·22 ± 0·42 | 3·22 ± 0·12 | 2·98 ± 0·01 | 4·27 ± 0·45 | 4·55 ± 0·26 | 4·06 ± 0·07 | 4·11 ± 0·29 |
| Concn. of collagen (g/100 g) | 5·6 ± 0·1 | 3·6 ± 0·6 | 3·1 ± 0·1 | 3·2 ± 0·1 | 2·7 ± 0·2 | 4·2 ± 0·3 | 3·4 ± 0·3 | 2·9 ± 0·2 |
| $l_1$ (mm) | 4·7 ± 0·2 | 10·6 ± 0·8 | 12·0 ± 0·1 | 11·6 ± 1·0 | 13·6 ± 1·6 | 7·6 ± 0·5 | 10·3 ± 2·3 | 13·1 ± 0·9 |
| $l_0$ (mm) | 5·5 ± 0·3 | 12·1 ± 1·2 | 13·7 ± 0·3 | 12·9 ± 1·1 | 15·4 ± 1·6 | 8·1 ± 2·4 | 11·6 ± 2·5 | 16·0 ± 1·0 |
| $10^3\ K/l_0$ | 1·09 ± 0·02 | 1·85 ± 1·03 | 1·24 ± 0·11 | 1·95 ± 0·16 | 1·52 ± 0·46 | 1·68 ± 1·08 | 2·59 ± 0·94 | 3·59 ± 0·52 |
| Stress at $l_0$ (g/mm² collagen) | 110 ± 3 | 263 ± 9 | 298 ± 16 | 303 + 26 | 236 ± 52 | 127 ± 43 | 200 ± 40 | 274 ± 25 |
| Circumference at break (mm) | 7·3 ± 1·3 | 17·6 ± 1·9 | 17·5 ± 0·6 | 16·9 ± 1·4 | 20·3 ± 2·3 | 14·3 ± 1·9 | 17·3 ± 5·2 | 23·2 ± 1·6 |
| Stress at break (kg/mm² collagen) | 1·34 ± 0·39 | 1·44 ± 0·18 | 2·02 ± 1·07 | 2·10 ± 0·62 | 1·55 ± 0·10 | 1·29 ± 0·13 | 1·61 ± 0·10 | 1·36 ± 0·12 |

The estimate of variation is the standard error of the mean. There were two rats in each group except the control and 10·0 mg *Std* (sample *C*) which had three. Animals were treated as in Table 6. The meanings of $l_1$, $l_0$, and $K$ are given in the text. g-p u., see Table 7.

force exerted by incorporating a spring gauge so that the probe can be pushed in with a standard pressure. This type of test is similar in principle to our first type (examination of the relation of circumference to tension) but the lack of a measure of force or time during which it is applied makes the results difficult to interpret with certainty. In particular, it is not clear to what extent the activity of smooth muscle affects this test. However, it seems probable that the probe would be arrested when the tissue had stretched to a circumference which in our experiments would lie somewhere near the beginning of the relatively flat part of the length-tension curves. Using this method Graham & Dracy (1952), Zarrow, Neher, Sikes, Brennan & Bullard (1956), Steinetz, Beach & Kroc (1956) and Kroc *et al.* (1959) have found that relaxin increases the 'dilatability' of the cervix in sow, rat and mouse. That relaxin has an effect on the cervix must therefore be regarded as clearly established. The smaller effect of oestrogen is less clear. Thus Smith & Nalbandov (1958) recorded a constriction of the cervical canal by oestrogen in the sow. Such a finding with the probe test might be the result of swelling of the inner lining only, and it is difficult to distinguish the various possibilities without more information on the forces used and time for which they acted. A number of observations on the effects of relaxin on the human cervix are recorded in the literature but the results have been so variable that it seems premature to discuss the subject. It is worth while to point out that in some of the experiments in which no effect was found only short periods of treatment (hours) were used. Experiments on animals indicate that much longer treatment is needed to produce effects.

*Nature of effect of hormones on the cervix*

The effect of oestradiol alone was to increase the weight of the cervix to about the normal non-pregnant level. The total quantity of collagen in the cervix increased proportionately less than weight so that concentration fell. This confirms previous results with oestrone (Harkness *et al.* 1957). The principal effect of oestradiol, shown in physical tests, was to cause an increase in the circumference of the collagenous framework, and this resembled the change in total collagen in that it was less extensive and took place more slowly than increase in weight. Thus the increase in total collagen content and in circumference appear to be related in both extent and time course. This suggests that both changes have a common origin in simple growth in size of the connective-tissue framework without change in its shape. Circumference is a linear dimension and should increase as the cube root of the total amount of tissue concerned if there is no change in shape. For animals other than those treated with relaxin combined with oestradiol, the circumference is, in fact, approximately

proportional to the cube root of the total collagen content (Fig. 8). We may conclude then that in these animals increase in circumference is to be accounted for by growth of the collagenous framework without change in shape. This conclusion, however, does not apply to the relaxin-treated (oestrogen-primed) animals, which show an increase in circumference with little increase in total collagen above the value found with oestradiol alone (Tables 4 and 6, Fig. 8). We may conclude that relaxin produces a change in the shape of the collagenous framework. In addition, it can increase the extensibility of the framework under prolonged loading.

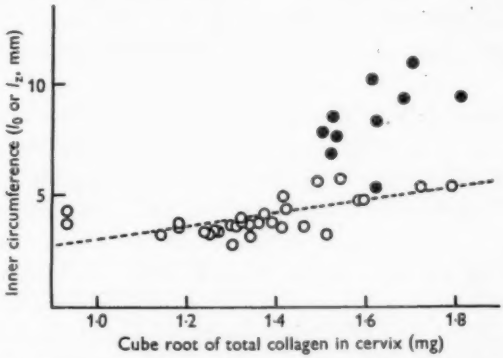


Fig. 8. Relation between inner circumference and total collagen content of horns. ●, animals treated with oestradiol and relaxin with or without progesterone; ○, other treatments, the dotted line going through the mean of abscissal and ordinate value for these, and having slope required for value of ordinate to be proportional to that of abscissa.

*Comparison of the effect of relaxin on the cervix with changes found in pregnancy.* During pregnancy in the rat the cervix becomes heavier in weight and its total collagen content is increased by about a half though the concentration is reduced (Harkness & Harkness, 1959*b*). The circumference of the connective-tissue framework also becomes greater and this is not accounted for by simple growth but involves change in shape. In addition to these changes there is an increase in the extensibility of the framework under prolonged loading (Harkness & Harkness, 1959*b*). All these changes can be produced by relaxin, though we have not so far found the latter to cause changes as great as those found at the end of pregnancy. The tests made on cervices of pregnant animals are not all exactly the same as those carried out in the present investigation, but a close enough comparison can be made to enable one to say that the maximum increase in circumference found in the present series of experiments corresponded to the value at about the 17–18th day of pregnancy. Kroc *et al.* (1959)

also found maximum values of circumference by the probe method to correspond with the values obtained by the same method at this time of pregnancy. It is impossible to say yet whether the inability of relaxin so far to reproduce the complete changes found in pregnancy in the cervix is the result of inadequate dosage or because other hormones are involved. That other hormones are involved is suggested by the fact that Kroc *et al.* (1959) found that relaxin given to rats oophorectomized in pregnancy would prevent fall in circumference of the cervix (probe method) and allow it to develop to the size found normally at the end of pregnancy. Factors other than relaxin appear to be of relatively greater importance in mice than in rats in producing the increase in the circumference of the cervix found by the probe test in pregnancy (Steinetz *et al.* 1956; Kroc *et al.* 1959).

*Comparison of effects of relaxin on cervix and symphysis pubis*

In the rat the symphysis pubis is scarcely affected by pregnancy or relaxin, so far as is known. In other animals, e.g. guinea-pig, two sorts of changes can be produced. The first is an oedematous swelling of the tissue which is detectable within a few hours of the time of administration (Abramowitz, Money, Zarrow, Talmage, Kleinholz & Hisaw, 1944; Zarrow & Money, 1948), but we did not find any evidence of such a rapid change in the cervix. The second change produced by more prolonged administration is a slow reorganization of the tissues of the symphysis in which bone and cartilage are absorbed and replaced by loose connective tissue, so that the originally short and compact structure is replaced by a long, loose ligament between the bone ends, which become widely separated. The changes in the cervix which we have investigated also take place slowly and clearly involve considerable reorganization of the tissue. For the actions of relaxin both on the symphysis and on the cervix, previous treatment with oestrogen is needed, relaxin alone having no effect.

Whether the same component of the relaxin preparation affects the cervix as the symphysis cannot be stated for certain. However, in our experiments with preparations of different potency by assay on the symphysis their potency correlated well with their effect on the circumference of the cervix. Our results are therefore compatible with the hypothesis that the same component of the preparation produces both effects.

## SUMMARY

1. The effect has been studied of previous treatment of spayed rats with oestradiol, progesterone and relaxin, individually and in combination, on the reaction of the excised cervix to tension, the tissues being extended under load between two parallel rods, one inserted through each cervical

canal. The differences between animals, detected by these tests, were (*a*) in the circumference of the connective-tissue framework and (*b*) in the extensibility of the framework under prolonged loading.

2. Oestradiol (10 $\mu$g cyclopentyl-propionate) caused an increase in the circumference of the cervix to approximately the normal non-pregnant level. This effect could be accounted for by simple growth of the whole cervix without change of shape.

3. Relaxin in beeswax and oil given to rats previously primed with oestradiol caused a further increase in circumference of the cervix which could not be accounted for by simple growth, but appeared to involve change in the shape of the collagenous framework. Relaxin also caused some increase in the extensibility of the cervix under prolonged loading. The changes produced resemble those found in pregnancy but were not as great as at the end of the latter. The maximum circumference corresponded to that found on 17–18th day of pregnancy. Relaxin caused a reduction in concentration of collagen in the tissue by an increase of material other than collagen and this effect also is similar to that found in pregnancy.

4. To produce changes of the type investigated the action of hormones for several days at least was required.

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# THE INFLUENCE OF PRE- AND POST-GANGLIONIC SECTION OF THE CERVICAL SYMPATHETIC ON THE INTRAOCULAR PRESSURE OF RABBITS AND CATS

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In the majority of previous studies on the reaction of the intraocular pressure to cervical sympathotomy, observations have been confined to manometric records of the pressure changes that occur immediately after the operation. The results of these short-term studies have been conflicting. Thus preganglionic section of the cervical sympathetic in rabbits has been reported to cause a fall in pressure (Wegner, 1866), no change in pressure (von Hippel & Gruenhagen, 1870) and to give results varying in one series from a slight fall to a slight increase (Greaves & Perkins, 1952) and in another series from no change to a striking rise in pressure (Davson & Matchett, 1951).

Tonometric studies in which the pressure in the eye has been recorded over longer experimental periods have yielded more consistent results. Hertel (1900) found that excision of the superior cervical ganglion of rabbits had no immediate effect on the intraocular pressure, but that after 1 hr the pressure on the operated side was reduced. Further tonometric observations showed that the pressure remained below normal for 3–4 days. Confirmation of this prolonged fall in pressure after ganglionectomy has recently been reported by Linner & Prijot (1955) and Lieb, Guerry & Ellis (1958). In a further study, Linner & Prijot (1957) found no change in the intraocular pressure 24 hr after preganglionic cervical sympathotomy. This striking difference between the effects of pre- and post-ganglionic section of the sympathetic was not, however, found in similar tonometric studies on cats (Jaffe, 1948); in these experiments both removal of the superior cervical ganglion and section of the preganglionic fibres caused a fall in the intraocular pressure lasting several days.

It is the purpose of this study to establish, by means of an accurate manometric technique, the time course of the changes in intraocular

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pressure following pre- and post-ganglionic section of the cervical sympathetic nerves in rabbits and cats. This was essential before the mechanisms underlying the pressure changes could be investigated (Langham & Taylor, 1959, 1960).

## METHODS

Adult rabbits (New Zealand white, weighing 2–3 kg) and cats (2–5 kg) of both sexes were used in these studies.

The intraocular and blood pressures were measured manometrically by the method described by Langham (1959) using Sanborn 267 B transducers, 150–1100 carrier preamplifiers and a 150 M rectilinear multichannel pen recorder. All manometric measurements were made on animals anaesthetized with a 25% solution of urethane in NaCl solution 0·9% (w/v) (1·2–1·7 g urethane/kg body wt.). No local anaesthesia was employed. The time which elapsed between the cannulation of the two eyes was less than 3 min and a further 10–15 min was allowed for equilibration before readings of the two intraocular pressures were taken.

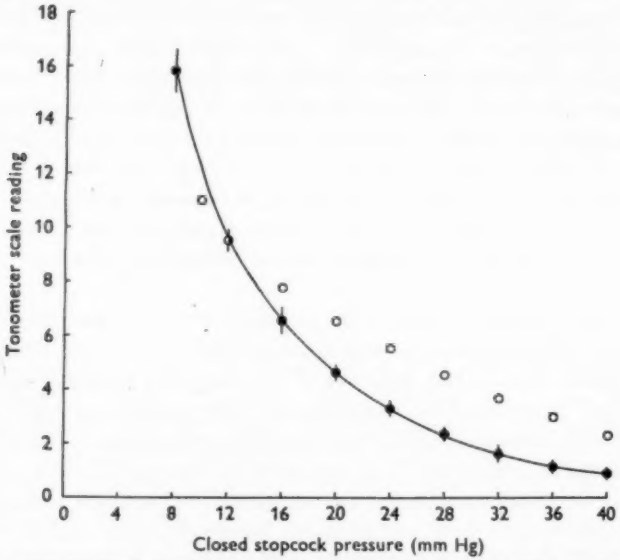


Fig. 1. Calibration of a standard Schiotz tonometer (7·5 g wt.) on a group of 16 rabbit eyes (●); each point indicates the arithmetic mean ± its standard error. For comparison, the 1954 calibration scale for a standard Schiotz tonometer (7·5 g wt.) on human eyes is included (○) (Friedenwald, 1954).

The intraocular pressure of conscious animals was determined by using a standard Schiotz tonometer (Dixey, London) weighted with 7·5 g. Rabbits were placed in canvas bags to restrain their movements and after instillation of 1–2 drops of a solution of amethocaine 1·0% (w/v) the tonometric readings were taken with the animal held on its side. The readings were converted to units of pressure by means of the calibration curve shown in Fig. 1. This was constructed from 'closed stopcock' manometric calibrations of sixteen dead rabbits' eyes *in situ*. The term 'closed stopcock' indicates that after the eye had been equilibrated at a known pressure the stopcock connecting the cannulated eye with the manometer was closed and a tonometric reading was then made (Perkins & Gloster, 1957).

Section of the preganglionic cervical sympathetic nerve and extirpation of the superior cervical ganglion was carried out on animals anaesthetized with pentobarbitone sodium (60 mg/kg) given intravenously, except in those animals in which intraocular pressure was to be measured either immediately or within 1 hr of the operation. In the latter urethane anaesthesia was used throughout the whole experiment. Section of the nerve was made 4–5 cm proximal to the ganglion.

The protein content of the aqueous humour was estimated semi-quantitatively by the addition of an equal volume of an 8% solution of trichloroacetic acid. Samples of normal aqueous humour of rabbits give a very faint turbidity under these conditions (10–40 mg protein/100 ml.). The aqueous humour of cats gives barely perceptible to slight turbidity (2–15 mg/100 ml.). In the present experiments the turbidity ranged from normal through moderate (40–100 mg/100 ml.) to actual flocculation (greater than 100–200 mg/100 ml.), and these three stages have been classified as +, ++, and +++ respectively.

## RESULTS

*Tonometric studies.* The effects of both preganglionic section and ganglionectomy on intraocular pressure have been studied tonometrically (Fig. 2). In these experiments measurements of intraocular pressure were

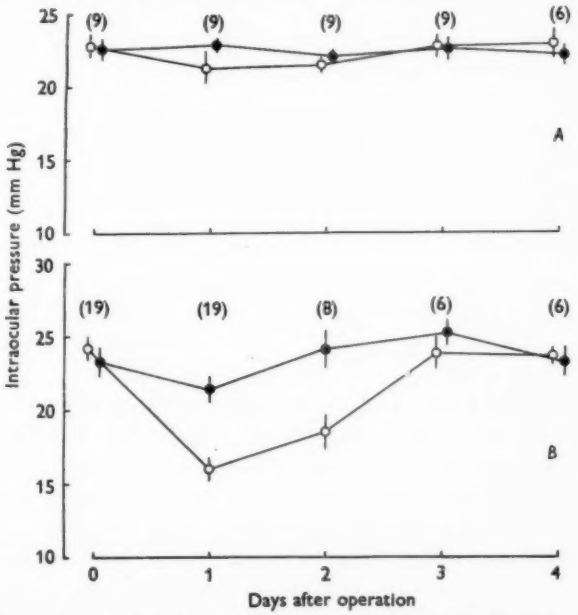


Fig. 2. The effect of unilateral section of the cervical sympathetic nerve (*A*) and superior cervical ganglionectomy (*B*) on intraocular pressure, measured tonometrically in rabbits. ●, mean pressure in the control eyes; ○, mean pressure in the sympathotomized eyes. The vertical lines represent one standard error above and below each point. The number of animals observed at each time is given in brackets. The pressures shown at zero time are those found immediately before sympathotomy.

not made until 24 hr after the operation, since preliminary studies had shown that repeated tonometry caused a striking rise in the protein content of the aqueous humour.

Of the nine rabbits in which unilateral preganglionic section of the cervical sympathetic was carried out, only one gave a fall in intraocular pressure on the operated side 24 hr after the operation. The mean values for the series show that there is no significant difference between the pressures in the two eyes during the 4 days following the sympathotomy (Fig. 2*A*).

TABLE 1. The immediate effect of pre- and post-ganglionic section of the cervical sympathetic on the intraocular pressure of rabbit (R) and cat (C) eyes. Symp., sympathetic section; G., ganglionectomy

| | Intraocular pressure (mm Hg) | | | Mean B.P. (mm Hg) | |
|---|---|---|---|---|---|
| Time after operation (min) ... | 0 | 2 | 10 | 0 | 10 |
| Operation | | | | | |
| Symp. (R) | 20·0 | 20·0 | 19·5 | 95 | 90 |
| Symp. (R) | 22·0 | 22·0 | 22·0 | 100 | 100 |
| Symp. (R) | 20·0 | 22·0 | 21·0 | 110 | 100 |
| Symp. (R) | 13·0 | 12·5 | 11·5 | 50 | 50 |
| G. (R) | 24·0 | 25·0 | 24·0 | 90 | 90 |
| G. (R) | 20·0 | 24·0 | 25·0 | 100 | 105 |
| G. (R) | 21·5 | 24·0 | 23·0 | 100 | 100 |
| Symp. (C) | 20·0 | 20·0 | 20·0 | 115 | 115 |
| Symp. (C) | 28·0 | 26·0 | 26·0 | 140 | 140 |
| Symp. (C) | 24·0 | 24·0 | 24·0 | 130 | 130 |
| Symp. (C) | 17·0 | 19·0 | 17·0 | 95 | 95 |
| Symp. (C) | 22·5 | 24·0 | 23·0 | 120 | 123 |
| G. (C) | 24·0 | 25·0 | 35·0 | 110 | 110 |
| G. (C) | 24 0 | 24 0 | 24·0 | 130 | 130 |
| G. (C) | 17·0 | 19·0 | 17·0 | 95 | 95 |
| G. (C) | 19·0 | 20·0 | 21·0 | 95 | 95 |

It is clear from Fig. 2*B* that 24 hr after excision of the superior cervical ganglion the intraocular pressure on the operated side ($16{\cdot}0 \pm 0{\cdot}7$ mm Hg, 19 eyes) is considerably less than that on the control side ($21{\cdot}3 \pm 0{\cdot}6$ mm Hg, 19 eyes). The results also suggest that there is a small fall in intraocular pressure in the control eye 24 hr after ganglionectomy. The value found here for intraocular pressure on the operated side at 24 hr is higher than that reported by Linner & Prijot (1957), and Lieb *et al.* (1958) in their tonographic studies, but is in very good agreement with the results of our manometric experiments. However, in agreement with these authors, it was found that the pressure in the operated eyes returned to normal by the third or fourth day.

*Manometric studies.* The results of pressure measurements taken at different time intervals after both preganglionic section and ganglionectomy in rabbits and cats are given in Tables 1 to 5.

The immediate reaction of the eye to these operations varied in both species and is illustrated by the manometric records of two typical

experiments shown in Figs. 3 and 4. In some animals the pressure in the eye gradually fell, but in others a sustained rise in pressure took place. The magnitude of these pressure changes was, however, with one exception small (less than 5 mm Hg) and of a transient character, for the pressures in all eyes began to return towards the initial values within 20 min. In the remaining animals no change took place after nerve section or ganglionectomy (see Table 1). In four experiments in which the pressures in the two eyes were measured during unilateral ganglionectomy no pressure change occurred in the contralateral eye.

TABLE 2. The intraocular pressures of control (*C*) and experimental (*E*) eyes of rabbits measured manometrically after unilateral extirpation of the superior cervical ganglion. For explanation of protein scale see Methods

| Time after operation (hr) | *C* Intraocular pressure (mm Hg) | *C* Protein | *E* Intraocular pressure (mm Hg) | *E* Protein | B.P. (mm Hg) |
|---|---|---|---|---|---|
| 1 | 23·0 | + | 21·5 | ++ | 115 |
| 1 | 18·0 | + | 18·0 | ++ | 110 |
| 1 | 21·5 | — | 19·0 | — | 130 |
| 1 | 16·0 | ++ | 14·5 | ++ | 100 |
| 1 | 22·0 | + | 21·0 | + | 105 |
| 1 | 23·0 | + | 22·0 | ++ | 125 |
| 6 | 22·0 | + | 22·0 | + | 115 |
| 6 | 18·5 | + | 17·0 | +++ | 120 |
| 6 | 20·0 | + | 17·5 | ++ | 125 |
| 6 | 19·0 | — | 18·0 | — | 105 |
| 24 | 23·0 | — | 17·0 | — | 110 |
| 24 | 21·5 | — | 12·5 | — | 110 |
| 24 | 20·0 | — | 18·0 | — | 105 |
| 24 | 19·5 | + | 14·5 | ++ | 115 |
| 24 | 22·0 | + | 16·5 | + | 110 |
| 24 | 24·0 | — | 17·5 | — | 120 |
| 24 | 21·0 | — | 14·0 | — | 120 |
| 24 | 22·0 | + | 18·0 | + | 110 |
| 24 | 22·0 | + | 16·0 | + | 130 |

TABLE 3. The intraocular pressures measured manometrically and protein content of control (*C*) and experimental (*E*) eyes of rabbits after unilateral preganglionic cervical sympathotomy

| Time after operation (hr) | *C* Intraocular pressure (mm Hg) | *C* Protein | *E* Intraocular pressure (mm Hg) | *E* Protein | B.P. (mm Hg) |
|---|---|---|---|---|---|
| 1 | 19·5 | + | 17·5 | ++ | — |
| 1 | 13·0 | + | 12·5 | ++ | — |
| 1 | 18·0 | + | 15·0 | + | — |
| 6 | 17·5 | + | 17·5 | + | — |
| 6 | 21·0 | + | 19·0 | ++ | — |
| 6 | 22·0 | + | 22·0 | +++ | — |
| 6 | 17·0 | + | 17·5 | ++ | — |
| 24 | 20·5 | + | 20·5 | ++ | 115 |
| 24 | 20·0 | + | 20·0 | + | 110 |
| 24 | 22·5 | + | 22·0 | + | 120 |
| 24 | 21·0 | + | 22·0 | ++ | 130 |
| 24 | 20·0 | + | 20·0 | ++ | 115 |

These results differ from the observations of Davson & Matchett (1951), who found rapid and moderately large increases in pressure (greater than 5–10 mm Hg) in 26 out of 49 rabbits after preganglionic sympathotomy. Unfortunately no details are given in their paper of the initial pressures and it is difficult to decide in what way their experiments differed from those described in this paper. However, it is evident that the intraocular pressures of some of their animals were not normal, for in Figs. 3, 4, 5 and 6

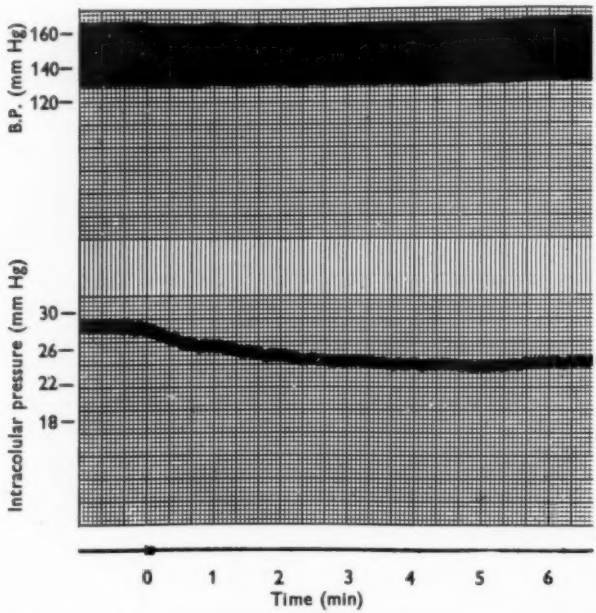


Fig. 3. The effect of preganglionic section of the cervical sympathetic nerve on the intraocular pressure (lower record) of an adult cat (4·4 kg) anaesthetized with urethane. The nerve was cut at $t = 0$ min. The femoral blood pressure is shown in the upper trace.

of their paper the initial pressures of pairs of eyes were (left/right) 35/19, 24/12, 18/7, 19/14 cm $H_2O$. This contrasts with the findings in this and other recent studies that the pressures in the two eyes of a pair are equal, rarely differing by more than 2 mm, and that the normal intraocular pressure in the rabbit is approximately 20 mm Hg (Langham, 1959).

After unilateral extirpation of the ganglion in rabbits the mean differences in the intraocular pressures of pairs of eyes (control–operated) at 1, 6 and 24 hr were $1{\cdot}25 \pm 0{\cdot}33$, $1{\cdot}25 \pm 0{\cdot}52$ and $5{\cdot}66 \pm 0{\cdot}65$ mm Hg. In contrast, there was no significant mean difference in the intraocular pressure of pairs

of eyes at 1, 6 and 24 hr after preganglionic cervical sympathotomy. The fall in pressure after ganglionectomy was confined to the operated side, for the mean pressure in the control eyes did not differ from normal. These observations are in general agreement with the findings of Linner & Prijot (1955, 1957) that 24 hr after ganglionectomy the pressure on the operated side was significantly lower than in the control eye. However, the mean fall in pressure of 6 mm Hg was less than the values of 10·2 and 15·4 mm Hg reported, respectively, by Linner & Prijot (1955) and Lieb *et al.* (1958) using tonometry.

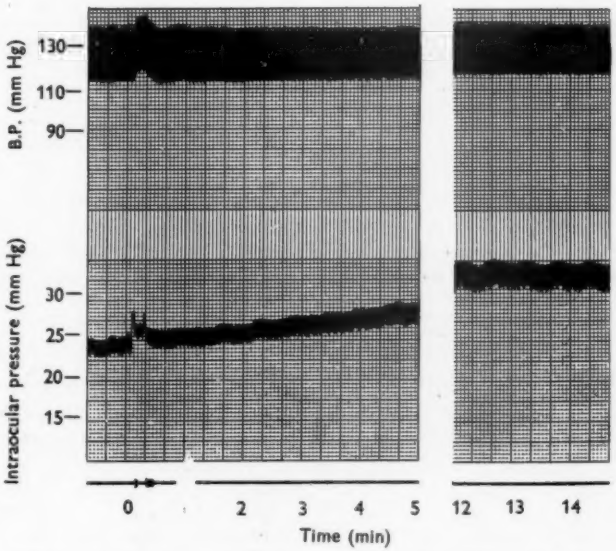


Fig. 4. The effect of excision of the superior cervical ganglion on the intraocular pressure of a cat (wt. 4 kg) anaesthetized with urethane. The ganglion was exposed before recording the initial intraocular pressure and was excised at $t = 0$ min.

The results of similar experiments on cats are recorded in Tables 4 and 5. Preganglionic section of the nerve caused no sustained effect on the intraocular pressure, but extirpation of the ganglion caused either a rise or a fall in pressure. These results differ from the tonometric observations of Jaffe (1948) that in cats both pre- and post-ganglionic section of the cervical sympathetic nerve caused a sustained fall in pressure.

In a number of these experiments the aqueous humours of the experimental and control eyes were withdrawn and the protein contents determined semi-quantitatively. The results of these analyses indicated that

both pre- and post-ganglionic section sometimes caused an increased concentration of protein in the aqueous humour, but there was no correlation between the protein content and the pressure change in the eye.

TABLE 4. The intraocular pressure measured manometrically and the protein content of control (*C*) and experimental (*E*) eyes of cats after unilateral extirpation of the superior cervical ganglion

| Time after operation (hr) | *C* | | *E* | | B.P. (mm Hg) |
|---|---|---|---|---|---|
| | Intraocular pressure (mm Hg) | Protein | Intraocular pressure (mm Hg) | Protein | |
| 1 | 19 | + | 21 | +++ | 100 |
| 1 | 16·5 | ++ | 17·5 | +++ | 115 |
| 1 | 23·0 | — | 20·0 | — | 120 |
| 1 | 16·0 | — | 19·0 | — | 105 |
| 1 | 20·5 | + | 18·5 | ++ | 95 |
| 24 | 14·1 | + | 8·6 | ++ | 100 |
| 24 | 40·0 | ++ | 45·0 | ++ | 210 |
| 24 | 15·0 | ++ | 23 | + | 135 |
| 24 | 23·0 | — | 20·0 | — | 120 |
| 24 | 16·0 | — | 19·0 | — | 105 |
| 24 | 20·5 | + | 18·5 | ++ | 95 |

TABLE 5. The intraocular pressures and protein content of control (*C*) and experimental (*E*) eyes of adult cats after unilateral preganglionic cervical sympathotomy

| Time after operation (hr) | *C* | | *E* | | B.P. (mm Hg) |
|---|---|---|---|---|---|
| | Intraocular pressure (mm Hg) | Protein | Intraocular pressure (mm Hg) | Protein | |
| 1 | 25·0 | + | 25·0 | + | 130 |
| 1 | 24·0 | + | 24·0 | + | 100 |
| 6 | 17·0 | ++ | 17·0 | ++ | — |
| 6 | 18·0 | + | 20·0 | + | — |
| 24 | 22·0 | + | 22·0 | + | 120 |
| 24 | 21·0 | — | 21·0 | — | 110 |
| 24 | 19·5 | + | 19·5 | ++ | 150 |
| 24 | 21·0 | + | 22·0 | ++ | 120 |

## DISCUSSION

In agreement with previous investigators we have found that the immediate effects of pre- and post-ganglionic section of the cervical sympathetic nerve on intraocular pressure are very variable. This is probably due to the variation from animal to animal in the relative values of sympathetic tone in different parts of the eye. The immediate response of intraocular pressure to sympathotomy is the sum of two opposing effects. First, denervation causes loss of tone in the extraocular smooth muscle and therefore tends to reduce intraocular pressure, and secondly denervation of the blood vessels with consequent vasodilatation increases the volume of fluid within the eye and tends to raise intraocular pressure. The results of the interplay of these two factors will vary with the level of sympathetic

tone and also with the ocular rigidity of the individual rabbits. Furthermore, since the sympathetic tone may vary with the experimental conditions, e.g. anaesthetic and ambient temperature, this may to some extent explain why different workers have obtained such conflicting results from apparently identical experiments. In view of this dependence of response upon the state of the eye, it is desirable but difficult to demonstrate that the eyes are in good physiological condition. In the manometric experiments carried out here on rabbits and cats there is remarkable consistency in the values for intraocular pressure in the control eyes. Ninety per cent of the values lie between 16 and 25 mm Hg. In addition, the close quantitative agreement between the results obtained by manometry and tonometry gives further support to the validity of these experiments.

The changes in pressure 1, 6 and 24 hr after section of the cervical sympathetic nerve were more consistent. This was to be expected, since any pressure changes resulting from alterations in the blood content of the eye or in extraocular muscle tone would be transient, being rapidly compensated for by a corresponding increase or decrease in the rate of drainage of aqueous humour. This means that the pressure changes observed when sufficient time has been allowed after sympathotomy for the eye to reach equilibrium, reflect principally, if not completely, alterations in the rate of aqueous humour formation and resistance to outflow.

The maximal pressure change of 6 mm Hg observed 24 hr after extirpation of the superior cervical ganglion in rabbits is significantly smaller than those reported by Linner & Prijot (1957) and Lieb *et al.* (1958). Both these groups of investigators reported that the intraocular pressure fell to within 1–2 mm Hg of the episcleral venous pressure. The discrepancy between these results and the present findings may well be due to the use of different anaesthetics and different methods for the measurement of the intraocular pressure.

The observations that the steady-state intraocular pressure in cats and rabbits was not modified after preganglionic sympathotomy, but underwent significant changes after extirpation of the ganglion, remain to be explained. The pressures recorded 1 hr after pre- and post-ganglionic section of the nerve did not differ significantly and suggest that the immediate effects of these operations on the eye are similar. However, the fact that the pressure in the eyes of the ganglionectomized rabbits continued to fall slowly and at 6 hr was only 50 % towards the change observed at 24 hr indicates that ganglionectomy led to a slowly developing effect on the intraocular dynamics. The nature of this change remains to be clarified.

SUMMARY

1. The intraocular pressure of rabbits and cats has been measured after pre- and post-ganglionic section of the cervical sympathetic nerve.

2. The immediate effect of these operations on the intraocular pressure varied but was transient, and the pressure difference between pairs of eyes 1 hour later did not exceed 2–3 mm Hg.

3. Preganglionic section caused no sustained effect on the intraocular pressure of rabbits or cats. Extirpation of the superior cervical ganglion in rabbits caused a slow fall in the steady-state pressure in the eye and this reached a maximum after approximately 24 hours. The pressure returned to normal within 3–4 days. In cats the effect of extirpation of the superior cervical ganglion, measured 1, 6 and 24 hours after the operation, was variable.

4. The significance of these results is discussed with reference to intraocular dynamics.

The authors wish to acknowledge their thanks to Sir Stewart Duke-Elder for his interest in this paper and to the Medical Research Council for defraying the cost of this study. We also wish to thank the Council to Combat Blindness, Inc., New York City, for a grant-in-aid towards buying part of the equipment used in this study.

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# THE INFLUENCE OF SUPERIOR CERVICAL GANGLIONECTOMY ON INTRAOCULAR DYNAMICS

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It is now well established that 24 hr after unilateral excision of the superior cervical ganglion in rabbits, the intraocular pressure on the operated side is reduced and that the pressure returns to normal by the third or fourth day (Hertel, 1900; Linner & Prijot, 1955; Lieb, Guerry & Ellis, 1958; Langham & Taylor, 1960). Since this is a change in the steady-state pressure it must be explained in terms of the three factors which govern intraocular pressure at equilibrium, namely rate of formation of aqueous humour, resistance to outflow and venous pressure. Linner & Prijot, and Lieb *et al.* have concluded from their tonographic studies that the observed fall in intraocular pressure is due to a marked decrease in the rate of formation of aqueous humour. In this paper more direct methods of measuring formation and resistance have been applied to this problem and our results indicate that 24 hr after ganglionectomy aqueous humour formation is normal and that the primary cause of the fall in intraocular pressure is decreased resistance to outflow. A preliminary report of this work has been published (Langham & Taylor, 1959).

## METHODS

Adult rabbits of the New Zealand White strain, weighing between 2·0 and 3·0 kg, were used throughout this study.

Excision of the superior cervical ganglion was carried out under pentobarbitone sodium anaesthesia (60 mg/kg), care being taken to expose the whole ganglion before excision to ensure complete ganglionectomy.

Intraocular pressure was measured manometrically with Sanborn 267B pressure transducers in conjunction with a Sanborn 150M rectilinear pen recorder. The technique for studying the effect of infusion of fluid into the anterior chamber on steady-state pressure has been reported by Langham (1959).

Fluorescein was estimated in the aqueous humour of the anterior chamber by the continuous fluorophotometric method described by Langham & Wybar (1954). This permits the accurate and continuous determination of the fluorescein concentration in both eyes throughout the course of the experiment without disturbing the physiology of the eye. The

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total concentration of fluorescein in the plasma was determined photometrically by means of the absorption at 435 mμ exhibited by fluorescein after treatment with concentrated hydrochloric acid. The concentration of free fluorescein was read from a conversion curve constructed from the results of a dialysis study of the binding of fluorescein by plasma.

The ascorbate concentration in the aqueous humour was estimated by the method of Mindlin & Butler (1938), the samples being deproteinized by centrifugation after the addition of an equal volume of 8% trichloroacetic acid. When it was necessary to saturate the animals with ascorbate, the schedule of intravenous and subcutaneous injections recommended by Becker (1956) was adopted. This gave a steady plasma level of 13–16 mg/100 ml. for several hours.

*Measurement of aqueous flow.* Flow of aqueous humour was estimated with fluorescein as test substance by a method similar in principle to that used by Goldmann (1950). An ideal test substance would be one which could be introduced into the aqueous humour under physiological conditions, the molecule of which would be large enough to preclude appreciable diffusion into the blood and adjacent tissues and yet not large enough to impede its free passage from the anterior chamber by bulk flow through the drainage channels. The transfer constant for the exit of such a substance from the anterior chamber ($k_{out}$) would be equal to the transfer constant for the loss of fluid from the anterior chamber by bulk flow ($k_{flow}$). Fluorescein being a substance of intermediate molecular weight approximates to these conditions and it will be shown that limits may be set to its deviation from this ideal behaviour.

A single intravenous injection of fluorescein (0·5 ml. 10% solution of the sodium salt) was administered to the rabbit under light pentobarbitone sodium anaesthesia. Frequent samples of blood were taken from a cannula in the femoral artery and the concentration of free fluorescein was determined. Frequent readings were also made of the concentration of fluorescein in the anterior chambers of both eyes during the course of the experiments which were continued for 2·5 to 3·5 hr. Since fluorescein enters the eye relatively slowly and since it is rapidly cleared from the plasma, the concentration in the aqueous humour passes through a maximum, usually between 30 and 60 min after the injection.

It is assumed that fluorescein enters the aqueous humour according to equation 1.

$$\frac{dC_a}{dt} = k_{in} \cdot C_p - k_{out} \cdot C_a, \tag{1}$$

where $C_a$ and $C_p$ are the concentrations of free fluorescein in the anterior chamber and the plasma, respectively, and where $k_{in}$ and $k_{out}$ are the transfer constants for the passage of fluorescein into and out of the anterior chamber. Equation 2 is derived from equation 1:

$$C_a = r \cdot k_{out} \cdot e^{-k_{out} \cdot t} \int C_p \cdot e^{k_{out} \cdot t} \cdot dt, \tag{2}$$

where the equilibrium constant

$$r = \frac{k_{in}}{k_{out}} = \left(\frac{C_a}{C_p}\right). \tag{3}$$

For each eye the curve of the aqueous humour concentration against time found experimentally is fitted with respect to the unknown constants $k_{out}$ and $r$, by means of equation 2 and the plasma fluorescein concentration curve. This is practicable, since the shape of the theoretical curves obtained in this way is sensitive to changes in $k_{out}$ and since $r$ merely alters the vertical axis. Figure 1 shows a typical fit and demonstrates the point just mentioned. This procedure yields $k_{out}$ for each eye.

$k_{out}$ has two components, since fluorescein may leave the anterior chamber either by bulk flow or by diffusion. Therefore

$$k_{out} = k_{flow} + k_{diff}, \tag{4}$$

where $k_{diff}$ is the transfer constant for diffusion between the anterior chamber and the plasma. For a given value of $k_{out}$, $k_{flow}$ will be greatest when $k_{diff}$ is zero; i.e.

$$k_{flow} \leqslant k_{out}. \tag{5}$$

The greatest value which $k_{diff}$ may attain is $k_{in}$, since under these conditions entry of fluorescein into the anterior chamber would be entirely by diffusion.

Therefore

$$k_{flow} \geqslant k_{out} - k_{in}, \tag{6}$$

and since

$$k_{in} = r.k_{out}, \tag{7}$$

$$k_{flow} \geqslant k_{out}(1-r). \tag{8}$$

The results have consequently been expressed in maximum and minimum values according to equations 5 and 8.

This mathematical derivation of aqueous flow is based upon the currently accepted theory of aqueous humour dynamics and it is implicit in this treatment that transport of fluorescein and water out of the aqueous humour is passive. No such assumption is made for transport into the aqueous humour, so that the equations are equally valid whether this transport is passive or otherwise.

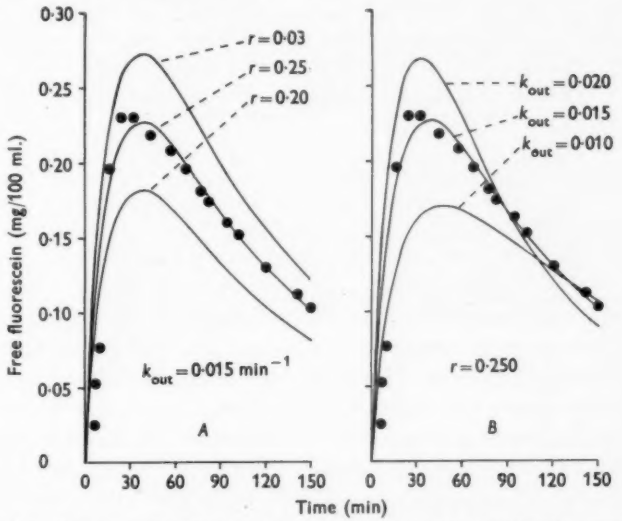


Fig. 1. A typical example of the way in which the observed values for the concentration of fluorescein in the anterior chamber are fitted with respect to $k_{out}$ and $r$ by means of the plasma concentration curve and equation 2. The experimental points are those for the ganglionectomized eye in Experiment 1, Table 1. *A* shows the effect of varying the equilibrium constant $r$ when $k_{out}$ is constant; *B* shows the effect of varying $k_{out}$ when $r$ is constant.

## RESULTS

*Effects of acetazoleamide.* The aim of these experiments was to determine the effect of acetazoleamide on the intraocular pressure and the outflow pressure (intraocular pressure–episcleral venous pressure) in the two eyes of individual animals 24 hr after unilateral ganglionectomy. The episcleral venous pressure has a mean value of 9 mm Hg in rabbits (Kornbluth & Linner, 1955) and is unaffected by either superior cervical ganglionectomy (Linner & Prijot, 1955) or acetazoleamide (Linner, 1956).

Table 1 summarizes the effects of an intravenous dose of acetazoleamide on the intraocular pressure of control and experimental eyes 24 hr after unilateral ganglionectomy. It is seen that the initial pressures (13·5–19·5 mm Hg) in the experimental eyes are significantly below normal (20–22 mm Hg) and that acetazoleamide reduces the pressure still further. The mean fall in the outflow pressure in the control and experimental eyes was $33{\cdot}5 \pm 2{\cdot}4\,\%$ (5) and $44{\cdot}0 \pm 3{\cdot}0\,\%$ (5), respectively. Figure 2 shows the result of a typical experiment and also demonstrates the increased intraocular pulse beat seen in the eye on the ganglionectomized side.

TABLE 1. The effect of acetazoleamide (25 mg/kg) injected intravenously on the intraocular pressure of control and experimental eyes 24 hr after unilateral extirpation of the superior cervical ganglion. The drug was given at time 0

| Control eye pressure (mm Hg) | | Experimental eye pressure (mm Hg) | | Mean B.P. (mm Hg) |
|---|---|---|---|---|
| 0 min | 15 min | 0 min | 15 min | |
| 21 | 17 | 13·5 | 11·0 | 114 |
| 23 | 16 | 17 | 13 | 110 |
| 21·5 | 17·5 | 15 | 12·5 | 130 |
| 23·0 | 20·0 | 19·5 | 16 | 90 |
| 20·0 | 16·0 | 15 | 12·5 | 120 |

*Rate of flow of aqueous humour.* In six experiments the rate of flow of aqueous humour in the two eyes was measured simultaneously 24 hr after unilateral ganglionectomy. The results are shown in Table 2. Both maximum and minimum values for $k_{\text{flow}}$ are given. It is clear that there is no significant difference between the values for the control and sympathotomized eyes, whether the maximum or minimum values are considered. The estimate of $k_{\text{flow}}$ in the control eyes, 0·0133–0·0116 $\text{min}^{-1}$, is in good agreement with that currently accepted for the rabbit (Kinsey & Barany, 1949).

The equilibrium ratio $r$ is higher on the operated than on the normal side by a factor of 1·7. This is the consequence of the increased permeability of the blood–aqueous barrier, a well known result of sympathotomy.

*Perfusion studies.* In order to obtain more direct evidence on the effect of ganglionectomy on the system draining aqueous humour from the eye, a series of infusion experiments was carried out. In these, physiological saline was infused into the anterior chamber at a known rate, and the new equilibrium pressure attained under this infusion was determined manometrically. Several measurements of this kind were made on each eye at different infusion rates and the equilibrium pressures were plotted against the corresponding rates of infusion.

Six infusion studies were carried out in this way and the results of four

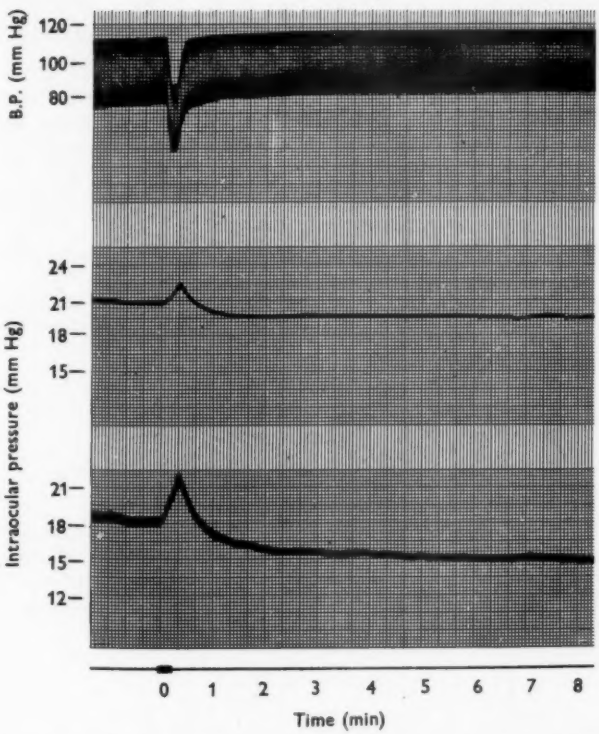


Fig. 2. The influence of an intravenous injection of acetazoleamide (25 mg/kg) at $t = 0$ min on the intraocular pressure of a rabbit 24 hr after unilateral excision of the superior cervical ganglion. Observations on the control and experimental eyes are shown in the middle and bottom records, respectively.

TABLE 2. The outflow constant ($k_{out}$) and the equilibrium constant ($r$) for fluorescein in the denervated and control eyes 24 hr after unilateral ganglionectomy in rabbits. $k_{out}$ and $k_{out}(1-r)$ give respectively the maximum and minimum estimates of $k_{flow}$. See equations 5 and 8

| | Ganglionectomized eye | | Control eye | | $k_{out}$ ratio Ganglionectomized / Control |
|---|---|---|---|---|---|
| Expt. No. | $k_{out}$ | $r$ | $k_{out}$ | $r$ | |
| 1 | 0·0150 | 0·250 | 0·0175 | 0·173 | 0·86 |
| 2 | 0·0140 | 0·079 | 0·0115 | 0·065 | 1·22 |
| 3 | 0·0175 | 0·253 | 0·0180 | 0·220 | 0·97 |
| 4 | 0·0170 | 0·350 | 0·0150 | 0·093 | 1·13 |
| 5 | 0·0075 | 0·150 | 0·0075 | 0·123 | 1·00 |
| 6 | 0·0175 | 0·130 | 0·0100 | 0·040 | 1·75 |
| Mean | 0·0148 | 0·202 | 0·01325 | 0·119 | 1·155 |
| S.E. of mean | 0·00156 | 0·0408 | 0·00173 | 0·0276 | 0·129 |
| | $k_{out}(1-r)$ | | $k_{out}(1-r)$ | | |
| Mean | 0·0117 | — | 0·0116 | — | — |
| S.E. of mean | 0·0012 | — | 0·0013 | — | — |

of these experiments are shown in Fig. 3. In these experiments initial infusion studies were made on the control and experimental eyes alternately. The curves relating pressure and infusions in the control eyes were similar to those previously obtained with the eyes of normal unoperated rabbits (Langham, 1955, 1959). In contrast, the curves for the denervated eyes showed distinct differences from the control curves. The initial pressures were lower and in five out of the six experiments the slopes of curves

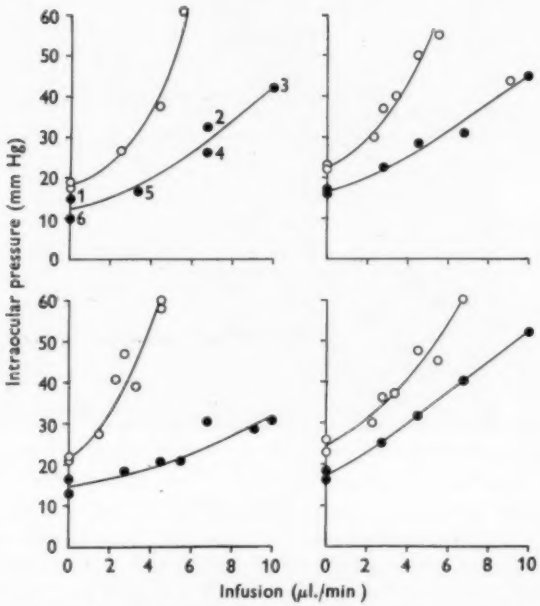


Fig. 3. The effect of superior cervical ganglionectomy on the response of intraocular pressure to the infusion of physiological saline (pH 7·4) into the anterior chamber. Each of the four graphs gives the results for a pair of eyes 24 hr after unilateral ganglionectomy. ●, denervated eye; ○, control eye.

relating pressure and infusion were significantly less than in the control eyes. The result on the sixth animal was in agreement with the rest of the series but the difference between the slopes of the two eyes was less marked (lower right-hand figure in Fig. 3).

*Ascorbic acid analyses and blood flow.* The concentration of ascorbic acid in the aqueous humour is dependent on the amount brought to the site of secretion in the ciliary processes (Linner, 1952; Langham, 1955). Consequently, provided the transfer mechanism is not saturated with ascorbic acid the concentration in the aqueous humour can be used to derive a measure of the blood flow through the ciliary processes. Table 4

shows the results of analysis 24 hr after ganglionectomy. It is seen that in the rabbits not given supplementary ascorbate the concentration in the aqueous humour on the operated side was higher than on the control side by a factor of 1·12. This ratio is of the same order as that found by Becker & Linner (1952) 24 hr after preganglionic section of the sympathetic chain.

TABLE 3. The effect of superior cervical ganglionectomy on the ascorbate content of aqueous humour at plasma ascorbate levels above and below saturation level in rabbits. These are compared with the effect of preganglionic section of the cervical sympathetic under similar conditions, as reported by Becker & Linner (1952). The aqueous samples were taken 24 hr after the unilateral operation. The figures in brackets indicate the number of experiments in each series and standard errors of the means are shown

| | | Denervated eye | Control eye | Ratio Denervated / Control |
|---|---|---|---|---|
| Ganglionectomy | Unsaturated | (8) 24·3 ± 1·73 | 21·9 ± 1·16 | 1·12 ± 0·04 |
| | Saturated | (6) 58·0 ± 3·4 | 63·6 ± 2·76 | 0·91 ± 0·03 |
| Preganglionic section | Unsaturated | (42) 26·0 ± 0·81 | 23·5 ± 0·91 | 1·11 ± 0·01 |
| | Saturated | (18) 50·1 ± 1·14 | 49·8 ± 1·32 | 1·01 ± 0·01 |

TABLE 4. Sensitization to adrenaline. Rabbits were subjected to either superior cervical ganglionectomy or preganglionic section of the sympathetic chain on the left side. 24 hr later the pupil diameter was measured in both eyes, before and after intravenous injection of 10 $\mu$g adrenaline

| | Pupil diameter (mm) before adrenaline | | Pupil diameter (mm) after adrenaline | |
|---|---|---|---|---|
| | Left eye | Right eye | Left eye | Right eye |
| Ganglionectomy | 7·5 | 8·5 | 10·5 | 7·0 |
| | 6·5 | 6·5 | 9·0 | 7·0 |
| | 8·0 | 9·0 | 10·0 | 8·0 |
| | 9·0 | 7·5 | 10 5 | 7·5 |
| | 7·5 | 7·5 | 10·0 | 10·0 |
| | 7·0 | 7·0 | 12·0 | 8·0 |
| Preganglionic section | 7·0 | 8·0 | 7·0 | 9·0 |
| | 7·0 | 8·0 | 7·0 | 8·5 |
| | 6·0 | 8·0 | 7·0 | 9·0 |
| | 6·0 | 6·0 | 7·0 | 8·0 |
| | 6·0 | 6·0 | 6·0 | 7·0 |

In the second series, the plasma concentration was raised to 13–16 mg/100 ml. for 4 hr before removal of the aqueous humour. In these rabbits the concentration of ascorbate in the aqueous from the denervated eye was less than that from the control eye: the ratio of the two concentrations was 0·91.

*Sensitization to adrenaline.* Paradoxical pupillary dilatation is a well known consequence of ganglionectomy in rabbits (Cannon & Rosenblueth, 1949). Table 3 shows the effect of an intravenous injection of adrenaline on pupil diameter 24 hr after preganglionic sympathotomy and also after ganglionectomy. It is evident that there was no significant difference

between the diameter of the pupils before the administration of the adrenaline in the denervated and control eyes in the two series. However, in the ganglionectomized animals the pupil on the operated side was strikingly more sensitive to adrenaline than the control pupil, but sensitization was absent in the animals with preganglionic section of the sympathetic chain. In similar experiments it was found that the sensitization on the ganglionectomized animals was not evident at 6 hr.

## DISCUSSION

The purpose of the studies described in this paper was to elucidate the mechanism which brings about the striking fall in intraocular pressure 24 hr after excision of the superior cervical ganglion in rabbits. Both Linner & Prijot (1955), and Lieb *et al.* (1958) found that at this time the intraocular pressure had fallen to within 1–2 mm Hg of the episcleral venous pressure, which they reported to be 9–10 mm Hg in both denervated and control eyes. This value is within the normal range of values for unoperated rabbits. These findings preclude the hypothesis that the fall in intraocular pressure is caused by a change in episcleral venous pressure, and the problem is resolved into whether the effect is due to a decrease in the rate of aqueous formation or to a decrease in the resistance between the anterior chamber and the episcleral veins or to a combination of both. The above authors determined the facility of outflow in rabbits 24 hr after unilateral ganglionectomy by tonography (Lieb *et al.* 1958) and by perfusion of the enucleated eyes (Linner & Prijot, 1955). In both cases the results on the control and experimental eyes were the same, and they concluded that aqueous humour formation had been greatly reduced; and Lieb *et al.* (1958) calculated the reduction to be more than 80 %.

We have found (Langham & Taylor, 1959), using both tonometry and the more direct method of manometry, that 24 hr after ganglionectomy the intraocular pressure fell to 15 mm Hg from its normal value of 20–22 mm Hg. If it is accepted that the episcleral venous pressure is unchanged, these results indicate that the outflow pressure has fallen by about 50 %. Therefore, unless there is an increase in outflow resistance the maximum possible fall in the rate of formation of the aqueous humour under these conditions is also 50 %.

The fluorophotometric studies indicate that the rates of flow of the aqueous humour in the eyes of individual rabbits after unilateral ganglionectomy were similar and within normal limits. A decrease of 50 % in aqueous humour flow would have been readily discernible by this technique and it must therefore be concluded that the decrease in intraocular pressure after ganglionectomy involved changes in the drainage system of the eye.

The results of the fluorophotometric studies are supported by the

observations that acetazoleamide, which is known to depress aqueous humour formation, caused a fall in the intraocular pressure of eyes already hypotensive as a result of ganglionectomy. Under these conditions acetazoleamide caused a similar proportionate decrease in the outflow pressure of the normal and hypotensive eyes. It is difficult to believe that this result would have been achieved if secretion of the aqueous humour had been depressed 50–80 % as a result of ganglionectomy, whereas the result is consistent with the conclusion that aqueous flow was similar in the two eyes.

Direct evidence that the outflow system is modified by ganglionectomy was seen in the results of the perfusion studies. It is clear that the slopes of the curves relating steady-state pressure with rate of infusion are mainly dependent upon the resistance to outflow, and that a gross change in the latter will manifest itself as a change in the slope. Some caution is, however, necessary in extrapolating these results back to the point of zero infusion, for the slope cannot under all circumstances be accepted to indicate outflow resistance alone, since at low infusion rates in the normal eye the slope of the curve approaches zero, whereas in fact resistance to outflow at this point is known to be considerable. It is, however, difficult to interpret the markedly shallower slopes from the ganglionectomized eyes except as indicating a striking reduction in the resistance to outflow of aqueous humour.

In considering the fall in intraocular pressure it should be remembered that this effect is found only after ganglionectomy and not after preganglionic section of the sympathetic chain. It has been suggested to us that the difference between the effects of the two procedures might be due to the formation of an irritative lesion after ganglionectomy but not after preganglionic sympathotomy, and consequently that the fall in intraocular pressure is due to an excess of sympathetic excitation rather than to a deficiency. This appears improbable, since after ganglionectomy the pulse pressure in the denervated eye is greater than that in the control eye (see Fig. 2), despite the mean pressure in the denervated eye being low, an occurrence which of itself would tend to reduce pulse pressure. Secondly, the ascorbate concentration in the denervated eye is higher than that in the control eye (the ratio of the concentrations being 1·12:1). It has been argued (Linner, 1952; Langham, 1955) that since at normal plasma ascorbate concentrations the mechanism for secreting ascorbate into the aqueous is unsaturated, then a change in the blood flow through the secretory area (ciliary body) will bring about a change in the same direction in the aqueous ascorbate concentration. The rise in the aqueous ascorbate concentration 24 hr after ganglionectomy suggests that blood flow through this eye has been increased as a result of the operation. Furthermore, the

increase would appear to be of the same order as that found by Becker & Linner (1952) after preganglionic section of the sympathetic chain.

At a plasma concentration above approximately 3 mg ascorbic acid/100 ml., the secretion of ascorbic acid into the aqueous humour reaches a maximum. Under these conditions 24 hr after ganglionectomy the concentration of ascorbate in the aqueous humour of the denervated eye was less than in the control eye, the ratio of the mean concentrations being 0·91:1. This is consistent with an increase in the rate of flow of aqueous humour in the experimental eye. However, the level of ascorbate in both eyes was very much greater than the plasma level and it is possible that the fall in concentration in the ganglionectomized eye was due to the increased permeability of the blood–aqueous barrier which accompanies pre- and post-ganglionic cervical sympathotomy.

Differences between the immediate effects on intraocular pressure of pre- and post-ganglionic sympathotomy have previously been reported. Von Hippel & Gruenhagen (1870) claimed that preganglionic section of the cervical sympathetic had no effect on intraocular pressure but that removal of the ganglion caused a rise in intraocular pressure. These authors stated that the difference was due to the preganglionic sympathetic fibre to the eye entering the sympathetic chain at the level of the superior cervical ganglion. This anatomical explanation is unacceptable, since no activity could be detected in the post-ganglionic fibres after section of the preganglionic trunk (E. S. Perkins & C. B. Taylor, unpublished), and also since the vascular changes appear to be the same for both operations. The difference between the effects of the two procedures is then the more perplexing, since apparently in both cases there is an equal and complete loss of sympathetic excitation.

There remains, however, the possibility that the differences between the action of pre- and post-ganglionic sympathotomy on the eye is linked with the phenomenon of sensitization, since this also occurs only after ganglionectomy. In this respect it should be noted that the onset of sensitization to adrenaline has approximately the same time course as the depression of intraocular pressure, both being scarcely detectable at 6 hr but very striking 24 hr after the operation. On the other hand, the parallelism ends after 24 hr, since pressure returns to normal within a few days while sensitization persists.

In conclusion, the evidence reported here points to a decrease in the resistance to outflow between the anterior chamber and the episcleral veins as the explanation for the fall in intraocular pressure observed 24 hr after ganglionectomy. The reason why this occurs after extirpation of the ganglion but not after preganglionic sympathotomy remains to be clarified.

SUMMARY

1. The cause of the fall in intraocular pressure found 24 hours after superior cervical ganglionectomy in rabbits has been investigated.

2. The rate of flow of the aqueous humour has been estimated by a fluorophotometric method and was found to be approximately the same in the eyes of individual rabbits 24 hours after unilateral ganglionectomy.

3. Evidence that ganglionectomy changed the resistance to outflow between the anterior chamber and the episcleral veins was obtained from perfusion studies on living eyes.

4. Analysis of the concentration of ascorbic acid in the aqueous humour indicated that ganglionectomy increased the blood flow through the eye.

5. Sensitization of the pupil to adrenaline, although absent 6 hours after ganglionectomy, was clearly demonstrated at 24 hours. This time course followed closely that of the pressure changes following ganglionectomy.

6. It is concluded that the fall in intraocular pressure 24 hours after ganglionectomy is due principally to a decreased resistance in the outflow system of the eye.

The authors wish to thank Sir Stewart Duke-Elder for his interest in this paper and Dr D. M. Maurice for suggesting the method of deriving the rate of aqueous flow from the fluorophotometric data. We are indebted to the Medical Research Council for defraying the costs of this study and also to the Council to Combat Blindness, New York City, for donating part of the equipment used.

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# MELANOPHORE-EXPANDING SUBSTANCES IN HUMAN BLOOD

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Shizume & Lerner (1954) detected in normal human blood a substance which darkened the isolated skin of *Rana pipiens*. Sulman (1956) also found such a material in human blood which darkened the tree frog *Hyla arborea*. These workers observed a higher titre of the active principle in the blood of abnormally pigmented patients, and this indicates its possible implication in the mechanism of pigmentation in human skin. Judging from the terminology used by Lerner it would appear that he believes the substance to be the melanocyte-stimulating hormone (MSH) from the donor's pituitary. Sulman (1956) did not so specifically identify the active principle: he called it 'Chromatophorotropic Hormone'. It seems premature to label it as a true hormone and in this paper it is referred to as melanophore-expanding substance (MES), and to avoid periphrasis the term MSH is used only in reference to Shizume's preparation.

Past attempts to associate higher concentrations of the active substance in the blood and urine of patients with pigmentary and other disease processes have had contradictory results (Shizume & Lerner, 1954). The untreated blood usually employed often proved toxic and the specificity of the assay methods is questionable. F. W. Landgrebe & H. Waring (unpublished) tried to improve this by partially purifying the extracts and assaying them on intact and on hypophysectomized *Xenopus laevis*, a test animal which is considered to be very specific. However, they failed to obtain uniform results; often they found extracts which darkened intact *Xenopus* but failed either to act on the hypophysectomized animal or to show a consistent dose–response relationship. Recently A. B. Lerner (personal communication to Landgrebe) has drawn attention to the relatively low sensitivity of the *in vivo* method as compared with the *in vitro* one used by him. Bentley & Hudson (1957), using *X. laevis* in both instances, claimed that the *in vitro* method is 100–150 times more

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sensitive than that *in vivo*. This is a false estimate based on concentration rather than on dose. From their results we calculate that the ratio of sensitivity is about 15. This comparative study is interesting, for it may explain the difficulties met by Landgrebe & Waring. However, it does not account for Sulman's positive *in vivo* result, unless it is assumed that Sulman's assay method is also more sensitive, a question which has probably not yet been reported.

The criterion applied by both Lerner and Sulman to evaluate the activity of the principle in blood is its capacity to darken amphibian skin. Such a change may be brought about by non-specific substances (Burgers, 1956). It was therefore decided to establish a few additional criteria, applicable to pituitary MES irrespective of their source and purity, and to examine how far the melanophore-expanding extracts from blood conform to them. The present paper embodies the results of these investigations.

## METHODS

The sources of MES were acetone-dried powders from the posterior pituitary lobes of ox and pig and from the anterior and posterior lobes of human beings. The former were commercial preparations, while the latter were from glands collected and dissected as quickly as possible after death. MES was obtained in each instance by extracting the powders with 0·25 % acetic acid according to the standard method (B.P. 1932; Landgrebe & Waring, 1941). Preparations of pituitary extracts having different degrees of purity were obtained by following, partly or fully, Landgrebe's method of purification of the hormone (Landgrebe & Mitchell, 1954).

Normal human blood was collected from the cubital vein and inert matter immediately precipitated with two volumes of acid acetone (25 ml. 0·005 N-HCl in 75 ml. acetone). It was quickly brought to the laboratory in iced containers and centrifuged. Four volumes of acetone were added to the clear supernatant and the precipitate, after settling during the night at −10° C, was collected by centrifugation, washed with acetone and dried over calcium chloride *in vacuo*. The active fraction was extracted with 0·25 % acetic acid (about 1 ml./ml. blood) and this solution was used for the test. Blood from patients was collected from different hospitals, brought to the laboratory in a frozen state and treated and tested similarly.

Three methods were employed to measure the activity: for assay *in vivo*, *X. laevis*, both intact and hypophysectomized, was used (Landgrebe & Waring, 1944): *Hyla arborea* was also used for assay *in vivo* (Sulman, 1952), but the method was modified in that the response was assessed by observing the melanophore change in the skin of the leg and not the gross change of skin colour; for assay *in vitro*, skin of *Rana temporaria* was used, *R. pipiens* being unavailable in this country. The procedure was a modification of the assay method employed by Frieden, Fishbein & Hisaw (1948). The method of assay with *Xenopus laevis* entails injection of 0·2 ml. of extract into the dorsal lymph sac through the thigh muscles. The melanophores in the central part of the large web in the animal's foot are examined microscopically with a $\frac{2}{3}$ in. (1·7 cm) objective and 10 × eyepiece with constant and adequate illumination. Similarly *Hyla arborea* is injected in the dorsal lymph sac and the melanophores in the skin of the leg observed microscopically. The skin of *Rana temporaria* is immersed in a suitable Perspex cup containing 2 ml. of the solution under test. At one and the same time the expansion of the melanophores is noted microscopically and the reduction of transmitted light measured by means of a suitable galvanometer.

A comparative study of the different methods has been made by Main. The results are as yet unpublished but it can be reported that the *in vitro* method with *R. temporaria* has been found to be about 50 times more sensitive on a concentration basis and 10 times on a weight basis than the *in vivo* method with *Xenopus laevis*, and the sensitivity of the *in vivo* assay with *Hyla arborea* is greater than that with *Xenopus laevis*. It has yet to be seen whether this latter result is dependent on the smaller size of *Hyla arborea*.

## RESULTS

Figure 1 represents the time–response curves obtained by plotting the melanophore changes (Landgrebe & Waring, 1944) after injecting different doses of International Standard ox posterior lobe pituitary extract into the dorsal lymph sac of *Xenopus*. The maximum response obtained with

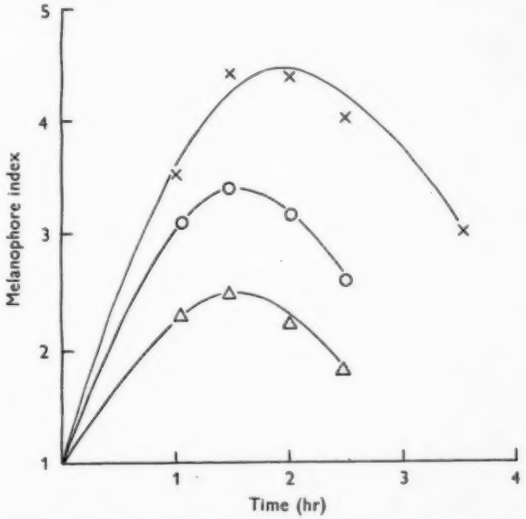


Fig. 1. Response curve obtained with different doses of international standard ox PLP; test animal, *Xenopus laevis*. ×, 2·5 μg; ○, 1·25 μg; △, 0·4 μg.

a certain dose of the substance is plotted as ordinate against the logarithm of the dose. All the points fall in a straight line, as is shown in Fig. 2.

Figure 2 shows the dose–response lines obtained with 0·25 % acetic acid extracts of ox, pig and human posterior lobe pituitaries. Human anterior lobe pituitaries were also extracted in a similar manner and the dose–response line is also shown. Two other dose–response lines are shown in the same figure, one from a partially purified pituitary preparation and the other from blood obtained from a grossly pigmented patient. The slopes of all these lines are similar.

Figure 3 shows the dose–response lines with *Hyla arborea* as a test

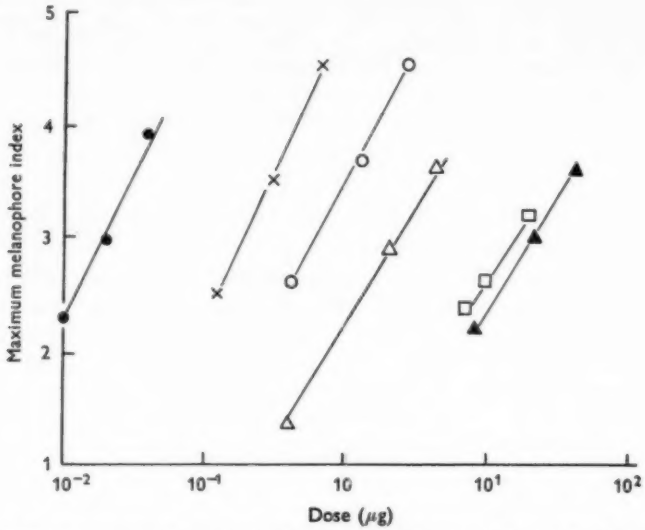


Fig. 2. Dose–response lines obtained with different preparations using *Xenopus laevis* as test animal. ●, Extract of a partially purified MES obtained from pig PLP; ×, extract of pig PLP; ○, extract of ox PLP (international standard); △, extract of human ALP; □, extract of human PLP; ▲, same from the acetone precipitate of blood obtained from patients. Semi-log. scale.

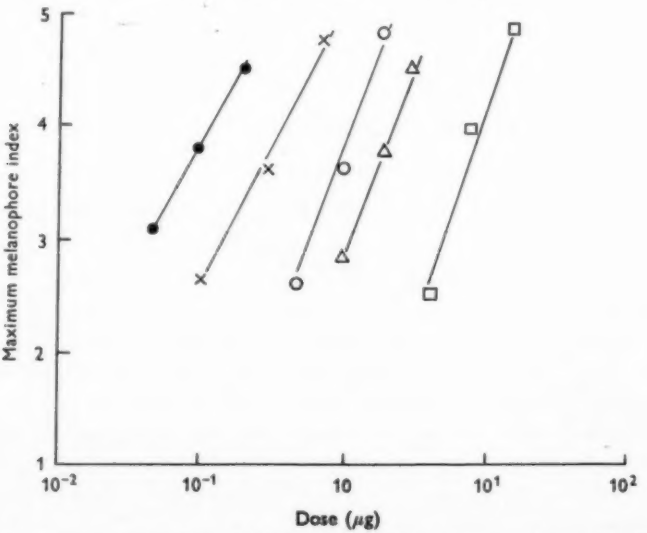


Fig. 3. Dose–response line obtained with different preparations with *Hyla arborea* as test animal. ●, Dixon's corticotrophin A1; ×, extract of pig PLP; ○, extract of ox PLP (international standard); △, extract of human ALP; □, extract of human PLP. Semi-log. scale.

animal. The dose–response line obtained with Dixon's corticotrophin A 1, which is known to possess melanophore-expanding properties (Dixon, 1956) is also included in the same figure. Again the lines are parallel.

Figure 4 shows the dose–response lines obtained by the *in vitro* method of assay. A dose–response line obtained from a preparation made from normal blood is also presented. It will again be seen that the different lines are similar in slope.

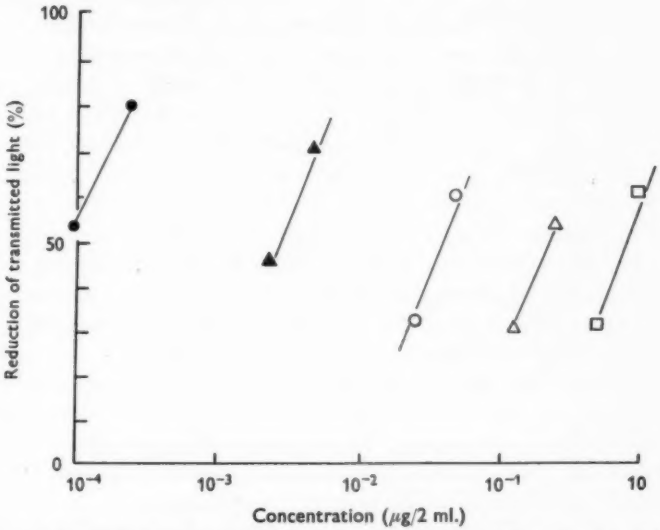


Fig. 4. Concentration–response lines obtained with different preparations on isolated skin of *Rana temporaria*. ●, extract of partially purified pig PLP; ○, extract of ox PLP; △, extract of human ALP; □, extract of human PLP; ▲, extract from acetone precipitate of normal human blood (the actual concentration is $10^{-6}$ times that shown in the graph). Semi-log. scale.

*Heat stability of melanophore-expanding substance.* The heat stability of melanophore-expanding preparations is well documented. Landgrebe & Waring (1941, 1944) and Landgrebe, Reid & Waring (1943) have shown that an acetic acid extract of ox posterior lobe pituitary retains its entire activity even after boiling for 3 min. We have found that this stability persists with increasing purification of the extract. Whether the activity of pituitary extracts is stable or not in a low concentration corresponding to the amount found in blood has not yet been determined. However, our blood extracts are relatively thermo-stable.

## DISCUSSION

Previous workers have recorded dose–response lines by both *in vivo* (Landgrebe & Waring, 1944; Thing, 1952; Bentley & Hudson, 1957) and *in vitro* (Shizume & Lerner, 1954; Bentley & Hudson, 1957) assay methods, but have usually confined themselves to a single species of amphibia and have drawn their lines from results obtained from only one preparation of MES.

We have recorded the dose–response lines obtained with pituitary extracts from different species in both *in vivo* and *in vitro* tests using three species of amphibia. We have found that with a particular assay method, *in vivo* or *in vitro*, and a particular *species* of amphibia the dose–response lines obtained with these *different melanophore-expanding extracts are parallel*. The parallelism is independent of the source of the pituitary or degree of purity of the extracts. This surprising finding not only indicates that the mechanisms of action of these pituitary substances on amphibian melanophores are probably similar, but also, when combined with the observation that an extract of blood from a pigmented patient gave a similar dose–response curve (Fig. 2), suggests that there may be in blood a MES of pituitary origin.

With preparations from normal blood we could not draw a line using *Xenopus laevis* or *Hyla arborea*, presumably owing to the relatively low sensitivity of the *in vivo* tests. It might have been possible if we could have more efficiently processed a larger volume of normal blood and removed the toxicity associated with the larger doses.

Figure 4 shows that by using isolated skin a dose–response line can be drawn for preparations from normal blood. This line is again parallel to those obtained with different pituitary preparations assayed in a similar manner. We have found that the MES from human blood is thermo-stable and we have further seen that this substance when injected into hypophysectomized *Xenopus* evokes a time response similar to that obtained with pituitary extracts. All these facts indicate that we are dealing with a substance which simulates pituitary preparations and may thus well be pituitary in origin.

Morris, Russell, Landgrebe & Mitchell (1956) suggested that in human pituitaries MES is elaborated in the anterior lobe and that the activity found in the posterior lobe may be due to post-mortem diffusion. For this reason it is interesting to compare the results we have obtained with the preparations from human anterior lobe, posterior lobe and blood. The dose–response lines obtained are all parallel to one another. However, the time–response curve obtained with the blood preparation simulates more closely that obtained with the anterior lobe extracts, that of the posterior

lobe being slightly different in that the change in 'melanophore index' (Hogben & Gordon, 1930) is exhibited after an initial time lag. This again indicates that the MES obtained by us from blood may be a pituitary substance having its origin in the anterior lobe. Thus, while our work adduces strong support for the claim that there is in blood a MES of pituitary origin, it does not definitely establish that the substance is a pituitary hormone. Indeed, as is indicated below it may even be adrenocorticotrophic hormone (ACTH).

Sulman (1952) claimed that ACTH preparations can darken *Hyla arborea*. A highly purified preparation from the pituitary, Corticotrophin A 1, also possesses melanophore-expanding activity equal to 1% of that of the pure melanophore-expanding polypeptide (Dixon, 1956). Winter, Brink & Folkers (1954) also found this activity in 'Corticotrophin B' preparation, which is a single substance by conventional physico-chemical tests.

Johnsson & Hogberg (1952) and Harris & Lerner (1957) have shown that the physico-chemical properties of the two polypeptides, MES and ACTH, are very similar except that the former has a smaller molecular weight. The melanophore-expanding property of ACTH may be due to one of two causes: either (1) the smaller polypeptide is intrinsic to the larger or is a contaminant difficult to remove from it; or (2) ACTH is an independent MES.

In view of the limitation imposed by the lesser availability of blood, as compared with urine, previous workers and ourselves have been forced to make only crude extracts of blood. The procedures used would also extract any ACTH which would give a positive response in our test if melanophore expansion is an inherent property of the ACTH molecule. It was thought that it might be possible to differentiate between the two polypeptides by drawing the dose–response lines. However, from our results even Dixon's Corticotrophin A 1 shows a dose–response line which has the same slope as other pituitary extracts (Fig. 3). It is thus difficult to claim that the active principle present in blood is a substance differentiated as a pituitary hormone, MSH. It is precisely for this reason that we feel justified in limiting the descriptive name of this active principle to 'melanophore-expanding substance'.

## SUMMARY

1. By means of acid-acetone a substance is extracted from human blood with an activity which simulates different pituitary melanophore-expanding preparations. The reasons for naming it 'melanophore-expanding substance' (MES) are discussed.

2. The various methods of assay of this substance are mentioned. The conclusions are that the *in vitro* methods are sensitive enough to measure the small amounts of this material present in normal blood and the *in vivo* methods are suitable for the measurement of the increased level found in the blood of persons with gross melanin pigmentation.

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# THE DISTRIBUTION OF RADIO-IODIDE IN THE SKIN OF THE RAT

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In previous studies it has been shown that about 30% of an injected dose of carrier-free radio-iodide is found in the skin of the rat 2–4 hr after injection. A concentration gradient for iodide, which can be abolished by thiocyanate, exists between skin and plasma (Brown-Grant & Pethes, 1959). An investigation has now been made into the distribution of radio-iodide in the skin 2–4 hr after injection.

## METHODS

Male albino rats, from an inbred laboratory colony, derived from the Wistar strain and maintained under the same conditions as in previous experiments (Brown-Grant & Pethes, 1959), were used. Carrier-free radio-iodide ($^{131}$I, obtained from the Radiochemical Centre, Amersham) was injected intraperitoneally in the doses given in the text. Tissue samples were dissolved in 3N-NaOH and their radioactivity measured in an M6 liquid counter.

The injection of high doses of $^{131}$I (40 $\mu$c or more) was carried out under ether anaesthesia, as were all surgical procedures. Potassium thiocyanate (10 mg/100 g body wt. as a 1% (w/v) solution) was also injected intraperitoneally.

Gross sections of the skin were prepared by freezing pieces, approximately 5 × 2 cm across and 4–6 mm thick, with solid carbon dioxide and cutting them freehand into 5–7 horizontal sections of roughly equal thickness. These were weighed while still frozen. Their $^{131}$I content was determined and the concentration (counts/100 sec/g tissue) and skin:plasma (S:P, (counts/100 sec/g tissue):(counts/100 sec/ml. plasma)) ratio for $^{131}$I calculated. Assuming an equal density for each horizontal slice, thickness would be directly proportional to weight, as the area of each slice was the same. On this basis the histograms showing the distribution of $^{131}$I in the skin were constructed.

In the autoradiographic studies, Ilford Nuclear Research Emulsions K5 and G5, both in gel form, were used. The emulsion was melted at 50° C and applied with a fine paint-brush to the slides, which were warmed to the same temperature. After cooling to room temperature the slides were exposed in the dark at 5° C in the presence of about 20 g of dried silica gel. Development was carried out with dilute Ilford ID-19 developer and fixation in 30% aqueous thiosulphate solution. Eisen & Harris (1957) have described a method for the preparation for autoradiography of tissues containing water-soluble isotopes; this involves rapid fixation in water-free acetone, followed by embedding in a water-soluble wax. Skin containing $^{131}$I was subjected to this treatment, but comparison with untreated skin showed that 50–60% of the activity was removed by the treatment. $^{131}$I histograms from pieces of

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skin fixed in acetone showed a loss of 80 % of the activity from the outer, epidermal, layer by comparison with fresh frozen skin, and were similar to those seen after KSCN administration (Text-fig. 2). Autoradiographs of sections from the blocks of treated skin confirmed that the $^{131}I$ had been lost from the tissues, and activity could be recovered from the water-soluble wax used for embedding. Conventional freeze-drying, followed by vacuum embedding in paraffin wax, was therefore used with all subsequent material.

In the first experiment an adult male rat weighing 220 g received 80 $\mu$c $^{131}I$. A first skin sample taken 2 hr later showed a skin:blood (S:B) ratio of 2·16:1, and an absolute $^{131}I$ content of 0·9 $\mu$c/g. The animal was then injected with KSCN and killed after one further hour, when the S:B ratio was 0·57, and the absolute skin content 0·4 $\mu$c/g. 5 $\mu$ sections of skin were used for autoradiography. After being dewaxed in petroleum ether for 10 min the slides were dried in air, and dipped once in a 1 % solution of nitrocellulose in ether:ethanol (1:1). 24 hr later the emulsion was painted over the sections, which were exposed for one or three half-lives, and examined by phase-contrast microscopy. The period elapsing between injection of $^{131}I$ and KSCN and the start of exposure was not more than one half-life. These sections showed no positive autoradiograph, an unexpected finding in view of the high initial $^{131}I$ content. A few blank slides were therefore coated with emulsion, and ribbons of sections mounted over the emulsion without dewaxing. After similar exposure periods an intense autoradiograph was obtained, clearly localized to the tissue within the ribbon. Evidently a loss of $^{131}I$ had occurred during dewaxing or dipping in nitrocellulose. This was investigated by measuring the radio-activity of ribbons of sections, mounted on slides, with a thin end-window Geiger counter before and after dewaxing for 2 min with petroleum ether. The dewaxing reduced the count rate by 30–40 %. Other dewaxing agents (xylene, benzene, toluene) caused even greater losses of $^{131}I$ from the sections. The leaching out of $^{131}I$ by the acetone and water-soluble wax treatment, and by non-polar dewaxing fluids after freeze-drying, prevented the preparation of autoradiographs in which the tissue section remained in contact with the emulsion.

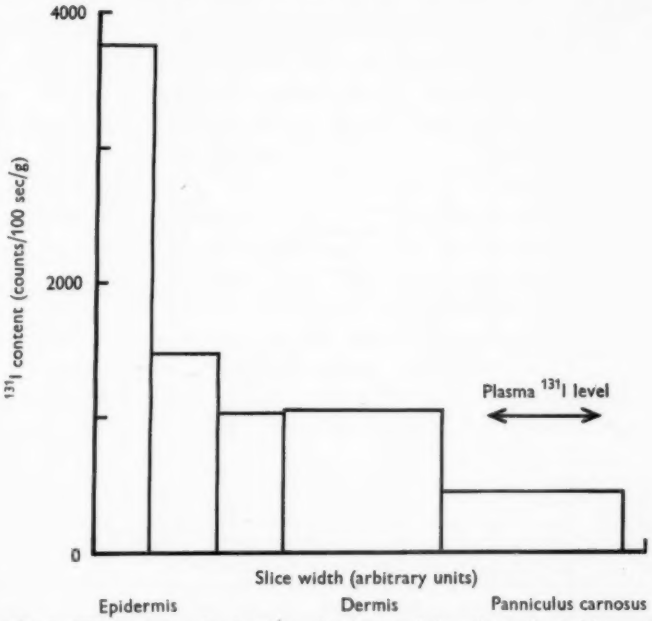
For the remaining experiments, autoradiographs were therefore prepared by two methods, 'direct' and 'sandwich'. In the first, or 'direct', method emulsion was spread on clean slides and allowed to dry: the waxed sections were then mounted on top of the emulsion, and gently flattened. After exposure the slides were placed in petroleum ether, which removed the waxed sections, and the emulsion was subsequently developed as usual. In the second, or 'sandwich', method emulsion was spread on clean slides, as before, and the sections mounted on a further series of slides. For exposure, an emulsion-coated slide was placed against a section-bearing slide and the two held firmly together with elastic bands. After exposure the two slides were separated, the one for development, the other for dewaxing and staining. The direct method gave autoradiographs with accurate resolution, but neighbouring sections had to be used for comparison; resolution was less clear with the sandwiches, but direct comparison between the section and its own autoradiograph was possible. The two techniques were essentially complementary and the information they gave agreed in all respects.

## RESULTS

### *Macroscopic skin sections*

Strips of skin were obtained from five adult rats 1–3 hr after the injection of 10–80 $\mu$c of $^{131}I$. The hair was removed with electric clippers. Hair obtained in this way up to 6 hr after injection contained no significant amount of $^{131}I$ and was therefore discarded. The strips of skin were frozen on plate glass to prevent curling. Histograms of the $^{131}I$ distribution in the skin were prepared as described under Methods. In each

case the outer layer showed the highest concentration of isotope, up to 8 times the plasma level. The middle layers, mainly dermal tissue, showed S:P ratios of 1–1·5:1; the inner layer of panniculus carnosus was always of lower $^{131}I$ content than the plasma (S:P ratio of about 0·6:1). Text-figure 1 shows a typical result. The mean skin $^{131}I$ content calculated from the data obtained from slices was within $\pm 10\%$ of the value obtained from fresh or frozen specimens of whole skin from the same animal.

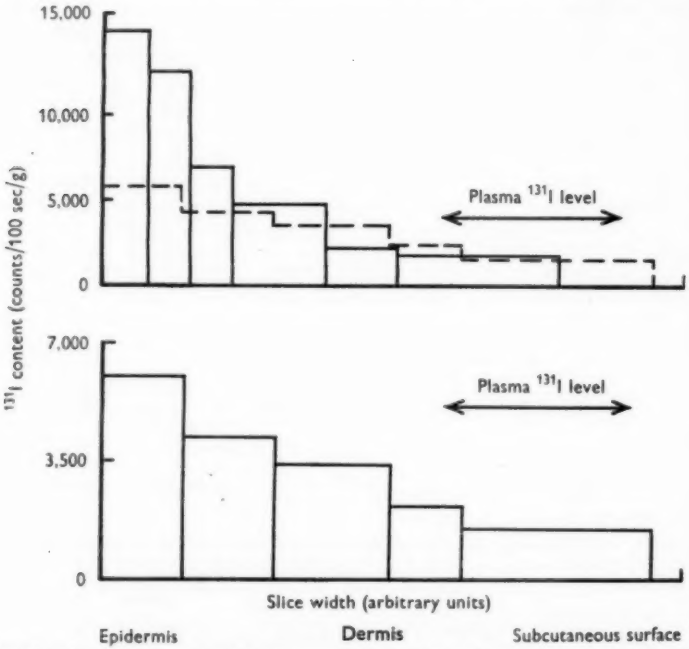



Text-fig. 1. $^{131}I$ concentration in successive horizontal sections of rat skin removed 2·25 hr after the injection of radio-iodide and cut freehand. S:P ratio for whole skin 1·78:1.

In two rats skin was removed from one flank 2 hr after injection of $^{131}I$ and a blood sample obtained. The animals were then injected with KSCN and allowed to recover. One hour later they were killed and skin was removed from a similar position on the opposite side of the body and a further blood sample obtained. The results of both experiments were the same; one is illustrated in Text-fig. 2. The major effect of KSCN is to reduce the $^{131}I$ content of the outer (epidermal) layers; the S:P ratio of the dermal layers falls to below 1·0:1 but the subcutaneous tissues are little affected.

These findings suggested that the region of high $^{131}I$ concentration was located in the epidermis. This had been suspected from the results obtained

when the S:P and S:B ratios for $^{131}I$ in rats of different ages were considered, together with the histological appearance of the skin. As is shown in Pl. 1 *A–D* the epidermal layers contribute a much greater proportion of the total skin thickness in the young rats than in the adult and also in the adult male as compared with the adult female. Quantitative studies were made as follows. Skin samples from the lumbar region of 2-day- and 10-day-old rats and adult male and female rats were fixed, dehydrated and em-




Text-fig. 2. $^{131}I$ concentration in successive horizontal sections of rat skin cut freehand. Above, skin removed 2 hr after injection of radio-iodide. Below, skin removed from same rat 1 hr after injection of KSCN and 3 hr after radio-iodide administration. Dotted line in upper histogram represents post-thiocyanate values. Note the preferential discharge of $^{131}I$ from the outer epidermal sections.

bedded in parallel. Measurements were made at four points on each of five sections of each type of skin. Values for relative epidermal thickness (as percentage of whole skin excluding subcutaneous muscle and fat) were $18{\cdot}8 \pm 0{\cdot}7$, $8{\cdot}6 \pm 0{\cdot}3$, $3{\cdot}4 \pm 0{\cdot}2$ and $2{\cdot}2 \pm 0{\cdot}1$%, respectively (mean ± standard error of the mean of 20 determinations). Typical S:B values were 5·7, 2·1 and 1·2 for 2-day-old, adult male and adult female rats in one series of experiments and 7·9, 4·5 and 3·0 for 2-day-old, 9-day-old and adult males in a second series.

*Autoradiographic studies*

These were carried out to obtain more exact information as to the location of the zone of high $^{131}I$ content, but were complicated, as has been discussed under Methods, by the fact that the isotope studied was present in a water-soluble form. Skin was taken from a 140 g adult male rat 2 hr after injection of 98 $\mu$c of $^{131}I$ and from the same animal 3 hr after the injection of $^{131}I$ and 1 hr after the injection of KSCN. The two samples of skin had $^{131}I$ contents of 4·6 and 2·2 $\mu$c/g and S:B ratios of 3·0 and 0·95, respectively. Skin samples were also obtained from a 2-day-old (5·4 g body wt.) and a 10-day-old rat (14·6 g body wt.) 2 hr after the injection of 14 and 23 $\mu$c of $^{131}I$, respectively. Skin contents were 14·3 and 6·8 $\mu$c/g and S:B ratios were 7·9 and 4·5.

Satisfactory autoradiographs (a.r.g.s) were obtained by both the 'direct' and the 'sandwich' method. Pl. 2*B* shows the a.r.g. obtained from a section of skin placed directly on the emulsion, and Pl. 2*A* a stained section from the same portion of the block. There is a band of high-grain density which corresponds to the deeper layers of the epidermis. This characteristic epidermal band is present though less clearly defined in the sandwich a.r.g. (Pl. 4*A*, *B*) of adult skin obtained before KSCN injection. Skin removed 1 hr later, however, gave a.r.g.s which indicated that the activity was now distributed more or less uniformly throughout the whole skin (Pl. 4*C*, *D*).

The skin of the suckling rat differs from that of the adult (Pl. 1) in the greater absolute, as well as relative width, of the epidermis, the presence of down-growths of the lower layers of the epithelium in the skin of the 2-day-old animal and a dense population of newly formed hair follicles in the 10-day-old animal. A.r.g.s show the same band of high $^{131}I$ concentration in the lower layers of the epidermis (Pl. 3*A–D*). Localized areas of high-grain density also occur frequently throughout the dermis in these sections and appear to correspond to the position of hair follicles.

## DISCUSSION

A concentration of $^{131}I$ in the whole skin of the rat relative to the blood can be demonstrated in the first few hours after injection. Histograms of the $^{131}I$ content of thick horizontal sections of frozen skin have shown that it is in the outer, predominantly epidermal, section that the highest concentration of $^{131}I$ occurs. After thiocyanate administration the preferential concentration of $^{131}I$ by this layer is abolished and the S:P ratio of this layer approaches that of the deeper layers. Autoradiographic studies have confirmed that the region of high $^{131}I$ concentration is in the

epidermis, more precisely in the lower layers, and that the preferential concentration of $^{131}I$ here is abolished by thiocyanate.

The significance of the iodide concentrating mechanism in this situation is unknown. It is of interest that Treherne (1956), studying the penetration of skin by non-electrolytes, deduced from the results of his dynamic experiments that the barrier to free diffusion lay in the epidermis. He predicted and demonstrated that a labelled compound ($^{35}S$-thiourea), during its passage through the skin, was concentrated in the lower layers of the epidermis, the same region in which we have observed the concentration of $^{131}I$. Morel (1959), on the basis of his studies on sodium transport by the skin of the frog, has suggested that the kinetics of this process can best be explained on the hypothesis that in the region of the basal layer of the epidermis there exists a narrow band of cells with unique cell membrane permeabilities and markedly different electrolyte content from the surrounding tissues. The phenomenon of iodide concentration may be related to these findings. An alternative possibility is that it is in some way connected with the biochemical changes occurring in the cells during keratinization, which would also account for the high concentration of $^{131}I$ in the region of the hair follicles in some of our a.r.g.s and may be connected with the rapid uptake of $^{131}I$ by the growing hair discussed elsewhere (Brown-Grant & Pethes, 1960).

## SUMMARY

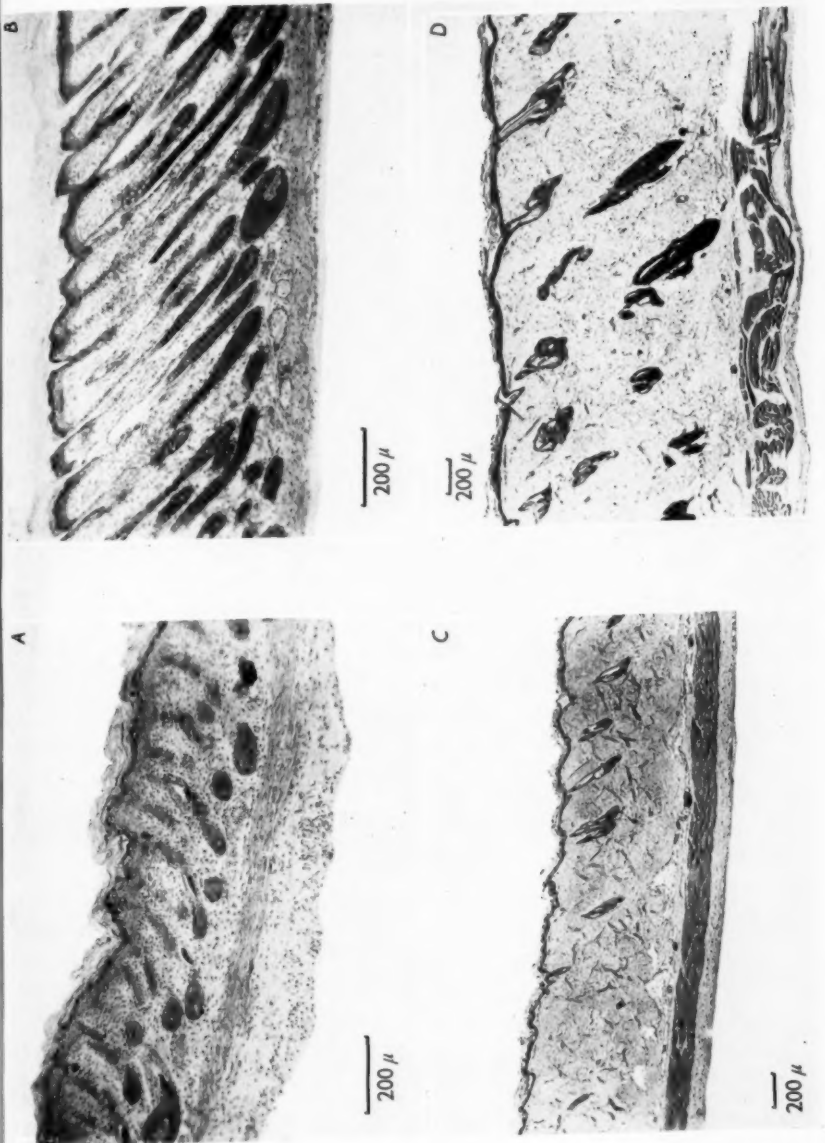
1. The existence of a skin:blood concentration ratio for $^{131}I$ greater than 1·0:1 in the first few hours after injection in suckling and adult rats has been confirmed.

2. Measurements of the $^{131}I$ content of macroscopic horizontal sections of frozen skin have shown that the highest concentration occurs in the outer epidermal layer and that $^{131}I$ is preferentially discharged from this region by thiocyanate.

3. Autoradiographic studies have shown that the $^{131}I$ that can be discharged by thiocyanate is located in the lower layers of the epidermis. A concentration of $^{131}I$ in the region of the hair follicles has also been observed.

4. Some possible explanations for the existence of an iodide-concentrating mechanism in the epidermis of the rat are discussed briefly.

Our thanks are due to Miss Heather Sorrill for her skilled technical assistance and to Miss Nita Dews for preparing the microphotographs.

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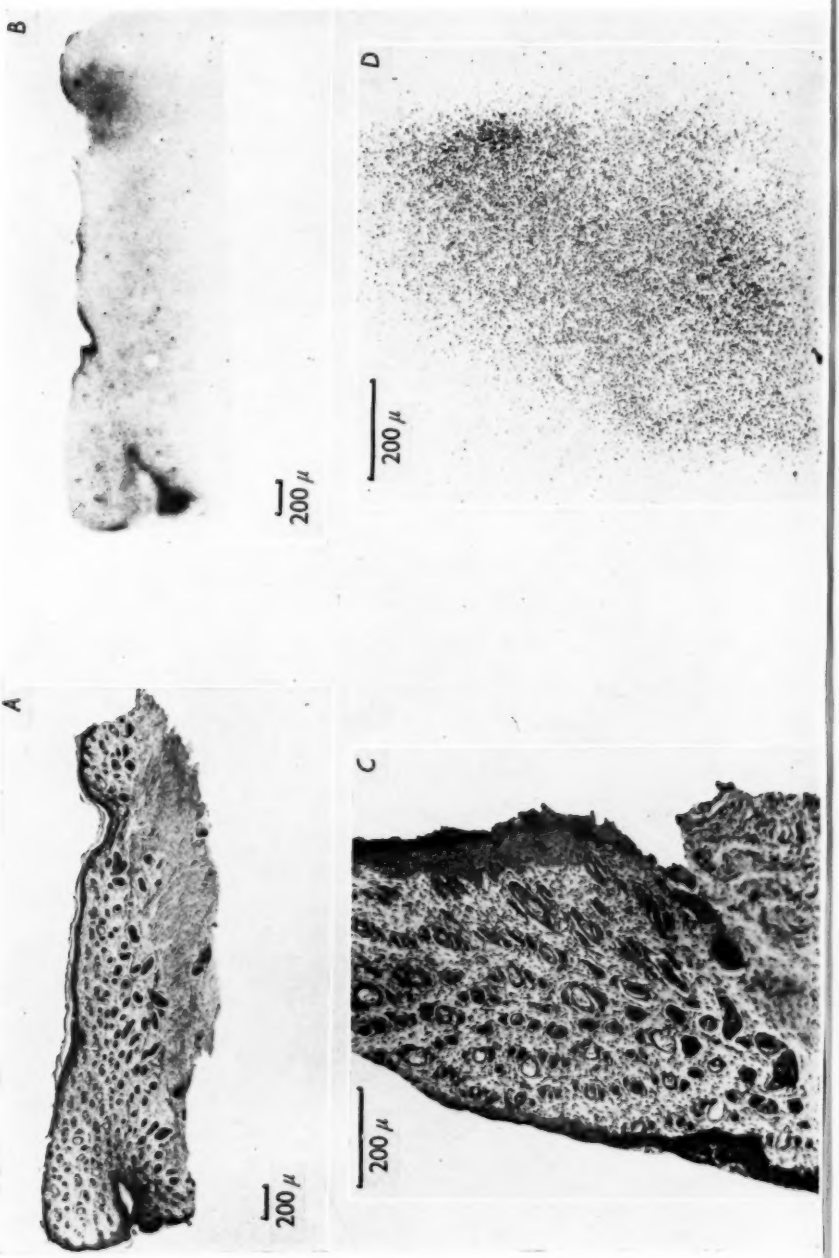


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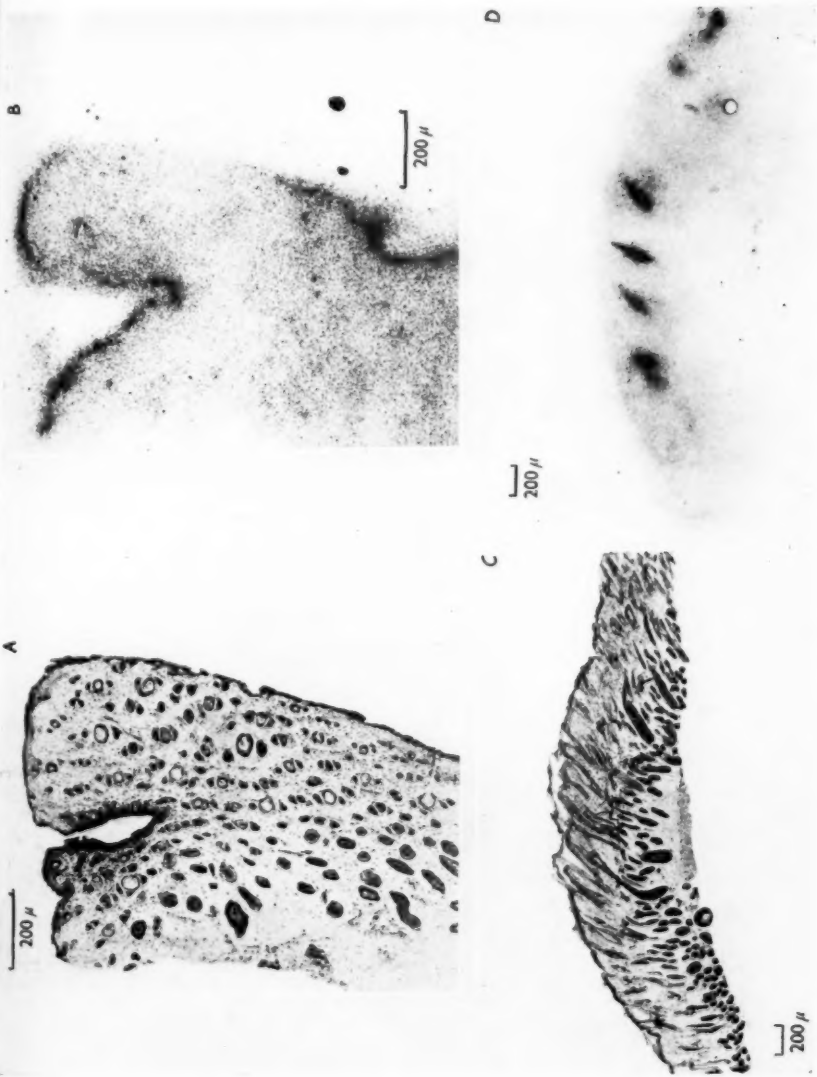


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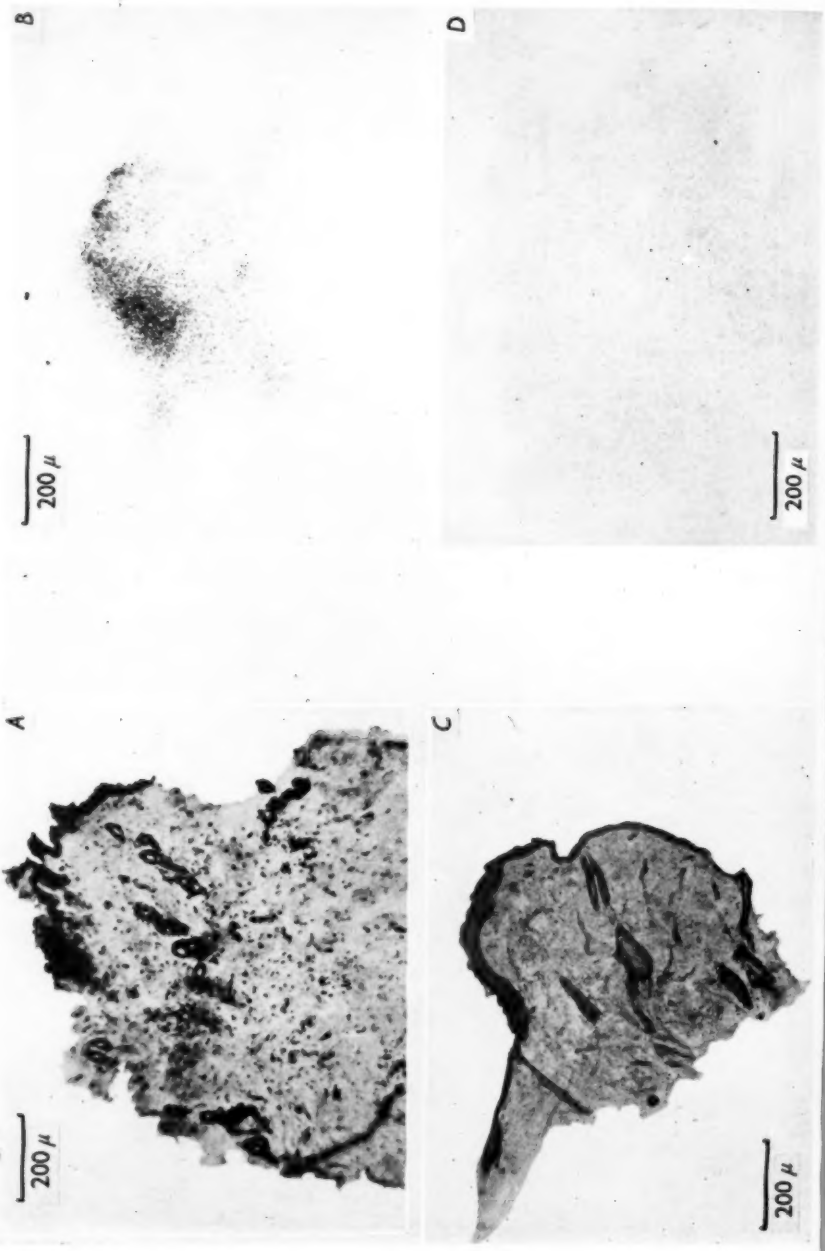


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## EXPLANATION OF PLATES

### PLATE 1

$5\mu$ sections of skin from lumbar region of rats of different ages. The tissues were processed simultaneously and stained with haematoxylin and eosin. Note relative proportions of epidermis and dermis and the density of hair follicles in the different skins. *A*, 2-day-old rat; *B*, 9-day-old rat; *C*, adult female rat; *D*, adult male rat.

### PLATE 2

*A*, section of skin from a 2-day-old rat 2 hr after injection of $^{131}I$. *B*, the corresponding direct a.r.g. from a section adjacent to *A*. Note the region of more intense blackening which corresponds to the epidermis. *C*, section and *D*, sandwich a.r.g. from skin of same animal as for *A* and *B*. The resolution is poorer with this technique, but a line of more intense blackening is seen at the right of the autoradiograph, corresponding to the position of the epidermis.

### PLATE 3

*A*, *B*, detail from section shown in Pl. 2*A*. Note absence of intense blackening in area where epidermis has been lost from the section. *C*, section of skin removed from a rat aged 10 days 2 hr after the injection of $^{131}I$, and *D*, the corresponding sandwich a.r.g. Note more intense blackening at the upper epidermal margin of the section and the blackening over the more superficial parts of some of the hair follicles. The deeper regions of the dermis containing the bulbs of the hair follicles show no localized concentrations of $^{131}I$.

### PLATE 4

*A*, section of skin from adult male rat, 2 hr after injection of $^{131}I$, and *B*, the corresponding sandwich a.r.g. Note intense blackening at edge which corresponds to the position of the epidermis. *C*, section of skin from same animal as for *A*, but removed 3 hr after $^{131}I$ injection and 1 hr after injection of thiocyanate, and *D*, the corresponding sandwich a.r.g. Note lower general density of graining and absence of any locally increased blackening.

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# THE UPTAKE OF $^{131}$I BY THE HAIR OF THE RAT

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(*Received* 4 *January* 1960)

In the first few hours after the injection of radio-iodide in the rat as much as 30% of the radioactivity is found in the skin. Thiocyanate given *before* the injection of $^{131}$I leads to a reduced skin content and a skin:blood (S:B) concentration ratio for $^{131}$I of less than one; when thiocyanate is given *after* the $^{131}$I, the skin content and S:B ratio are reduced as compared with control values, but not always to the level seen in animals pre-treated with KSCN (Brown-Grant & Pethes, 1959). These findings suggest that a part of the $^{131}$I present in rat skin as early as 2 hr after injection is in some form of organic combination or in some anatomical compartment from which it is no longer discharged by thiocyanate. This fraction could be the hair iodine. It has been shown that in the adult male rat 3–5% of an injected dose of $^{131}$I is still present in the skin 24 hr after injection and remains at this level for the next 10–12 days. The proportion appearing in hair removed by clipping gradually increases over this period (Brown-Grant & Pethes, 1959).

The incorporation of $^{131}$I into the hair of the rat has now been studied in more detail, with particular reference to the effect of chemical agents known to interfere with the concentration and organic binding of iodine elsewhere in the body.

## METHODS

The strain of adult male albino rats used, their housing conditions and diet, radioactive materials and methods of counting were the same as in previous studies (Brown-Grant & Pethes, 1959; Brown-Grant, Pethes & Rogers, 1960). In each experiment a suitable portion of the $^{131}$I solution injected was counted as an internal standard and results were expressed as a percentage of the injected dose. In short-term experiments in which KSCN was administered after the injection of $^{131}$I, the skin content of radio-iodide was also expressed as millilitres plasma equivalent; that is, the volume of plasma (in millilitres) having the same $^{131}$I content as the skin. This was necessary, since KSCN blocks the iodide-concentrating mechanism of the thyroid, jejunal glands and gastric mucosa. Under these circumstances the plasma concentration of $^{131}$I is higher in KSCN-treated animals than in controls after the same dose of $^{131}$I. Under these circumstances, therefore, expressing the skin content of radio-iodide in terms of millilitres plasma equivalent affords a better basis of comparison with control animals than expressing it as percentage dose. This has been discussed in a previous paper (Brown-Grant & Pethes, 1959).

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All results given in the text and in the tables are means ± standard error of the mean. The significance of differences between means was estimated by Student's *t* test and results are given as the probability (*P*) value obtained. 'N.S.' (not significant) indicates a value for *P* of greater than 0·05.

Potassium thiocyanate, A.R. (10 mg/100 g body wt. as a 1% (w/v) solution in distilled water), potassium perchlorate, L.R. (2 mg/100 g body wt. as a 0·5% (w/v) solution in distilled water) and 4-methyl-2-thiouracil (MTU) L.R. (10 mg/100 g body wt. as a fine suspension 50 mg/ml. in 0·01 N-NaOH) were injected intraperitoneally. In chronic experiments, KSCN 1% (w/v), $KClO_4$ 0·5% (w/v) or a saturated solution of MTU replaced the tap water normally supplied to the rats.

## RESULTS

### *The effects of thiocyanate and perchlorate*

Three groups of four animals (220–280 g body wt.) received 40 μc of radio-iodide by intraperitoneal injection. One group served as controls. The rats of the second group were injected with KSCN 45 min before and again 3 and 7 hr after the injection of $^{131}I$ and were given 1% KSCN solution to drink for the first 48 hr of the experiment (pre-treated group). Those of the third group received KSCN 3 and 7 hr after the $^{131}I$ and also drank KSCN solution for 48 hr (post-treated group). All animals were killed on the ninth day after injection. Hair, skin and thyroid gland were removed, weighed and their $^{131}I$ contents measured. The hair was removed from two parts (1) new-grown hair from the left flank from which hair had previously been clipped at the time of injection and (2) hair from the rest of the body, excluding the head and the ano-genital region. These two regions were also excluded in removing the skin. The results are given in Table 1. Pre-treatment with thiocyanate causes a significant reduction in

Table 1. The effect of thiocyanate, before or 3 hr after radio-iodide injection, on the distribution of $^{131}I$ in adult male rats 9 days later

(Four rats in each group. In all tables each value given is mean ± standard error of the mean.)

| | Group 1 controls | Group 2 Pre-treated KSCN | Group 3 KSCN at 3 hr | *t* test of significance of difference (*P* values). Comparison of Groups: 1 with 2 | 1 with 3 | 2 with 3 |
|---|---|---|---|---|---|---|
| Thyroid $^{131}I$ (% injected dose) | 3·16 ± 0·27 | 0·27 ± 0·07 | 0·73 ± 0·10 | < 0·001 | < 0·001 | < 0·02 |
| Skin $^{131}I$ (% injected dose) | 0·43 ± 0·08 | 0·08 ± 0·03 | 0·27 ± 0·13 | < 0·02 | N.S. | N.S. |
| Hair $^{131}I$ (% injected dose) | 1·45 ± 0·42 | 0·20 ± 0·04 | 0·51 ± 0·09 | < 0·05 | N.S. | < 0·05 |
| % dose/g hair | 0·45 ± 0·11 | 0·06 ± 0·02 | 0·16 ± 0·02 | < 0·02 | < 0·05 | < 0·05 |
| % dose/g new-grown hair | 1·25 ± 0·36 | 0·11 ± 0·01 | 0·30 ± 0·07 | < 0·05 | < 0·05 | = 0·05 |

the $^{131}I$ content of the thyroid, skin and hair and in the $^{131}I$ concentration in hair 9 days later (Group 1 compared with Group 2 in Table 1). The values for the post-treated animals of Group 3 fall between those of Groups 1 and 2. These findings indicate that the uptake of $^{131}I$ by the hair is dependent upon the action of an iodide-concentrating mechanism that can be inhibited by thiocyanate. In addition, the results obtained by delaying the thiocyanate administration until 3 hr after the injection of $^{131}I$ show that an irreversible uptake by the hair has already occurred at this time.

TABLE 2. The effect of perchlorate administration on the $^{131}I$ content of thyroid, skin and hair 9 days after injection of radio-iodide

(Five rats in each group)

| | Control | Perchlorate treated | $t$ test of significance of difference ($P$ values) |
|---|---|---|---|
| Thyroid $^{131}I$ (% injected dose) | $2{\cdot}03 \pm 0{\cdot}20$ | $0{\cdot}02 \pm 0{\cdot}01$ | $< 0{\cdot}001$ |
| Skin $^{131}I$ (% injected dose) | $0{\cdot}51 \pm 0{\cdot}14$ | $0{\cdot}05 \pm 0{\cdot}01$ | $< 0{\cdot}02$ |
| Hair $^{131}I$ (% injected dose) | $0{\cdot}52 \pm 0{\cdot}12$ | $0{\cdot}18 \pm 0{\cdot}02$ | $< 0{\cdot}05$ |
| % dose/g hair | $0{\cdot}25 \pm 0{\cdot}05$ | $0{\cdot}09 \pm 0{\cdot}01$ | N.S. |
| % dose/g new grown hair | $0{\cdot}77 \pm 0{\cdot}24$ | $0{\cdot}18 \pm 0{\cdot}03$ | $< 0{\cdot}05$ |

In a similar experiment the effect of pre-treatment with perchlorate on the $^{131}I$ content of hair was examined. Five control and five experimental animals were used (180–200 g body wt.). Perchlorate was injected 45 min before and 4 hr after the injection of $^{131}I$ and the animals drank 0·5% $KClO_4$ solution for 6 days. The results are given in Table 2 and show that perchlorate, like thiocyanate, reduces the uptake of $^{131}I$ by the hair. The expected decrease in the $^{131}I$ content of the thyroid glands of the treated animals as compared with the controls was seen in both these experiments.

*The effects of methyl-thiouracil administration*

By analogy with the thyroid, the $^{131}I$ that is already taken up by the hair and which cannot be discharged by thiocyanate might be present as organically bound iodine. The effect of MTU on the uptake of $^{131}I$ by skin and hair was therefore investigated.

In the first experiment four groups of three rats each (230–280 g body wt.) received 4 $\mu c$ of radio-iodide and were killed 3 hr later. The $^{131}I$ contents of plasma, thyroid and skin were determined. The four groups were (1) a control group injected with $^{131}I$ only, (2) a group injected with KSCN 2 hr after the injection of $^{131}I$, (3) a group pre-treated with MTU 45 min before $^{131}I$ injection and (4) a group receiving both MTU before and KSCN after the $^{131}I$ injection. Table 3 shows the results obtained. Plasma $^{131}I$ levels showed the expected rise after KSCN administration; the values for the thyroid indicated that organic binding of iodine in the thyroid and

the thyroid:plasma concentration gradient for $^{131}I$ had been abolished. Skin content, expressed either as percentage of the injected dose or as millilitres plasma equivalent, and the S:P ratio are not significantly affected by MTU (Group 1 compared with Group 3). The fall in S:P ratio and skin content as millilitres plasma equivalent after thiocyanate are significant as compared with the appropriate control group (Group 2 compared with Group 1 and Group 4 compared with Group 3). However,

TABLE 3. The effect of MTU given 45 min before and of KSCN given 120 min after the injection of radio-iodide on $^{131}I$ distribution 3 hr after injection in the rat

(Three rats in each group.)

| Group | Plasma $^{131}I$ (% dose/ml.) | Thyroid $^{131}I$ (% dose) | Thyroid: plasma ratio | Skin $^{131}I$ (% dose) | Skin:plasma ratio | Skin $^{131}I$ as ml. plasma equivalent |
|---|---|---|---|---|---|---|
| Control | 0·42 ± 0·02 | 3·08 ± 0·19 | 540 ± 33 | 23·7 ± 3·2 | 1·37 ± 0·12 | 56 ± 7 |
| KSCN at 120 min | 0·65 ± 0·07 | 2·04 ± 0·40 | 204 ± 41 | 16·8 ± 0·5 | 0·59 ± 0·03 | 26 ± 2 |
| Pre-treated MTU | 0·49 ± 0·05 | 0·19 ± 0·01 | 22·6 ± 1·5 | 21·9 ± 1·2 | 1·19 ± 0·06 | 45 ± 6 |
| Pretreated MTU, KSCN at 120 min | 0·62 ± 0·09 | 0·013 ± 0·001 | 1·45 ± 0·16 | 15·6 ± 2·7 | 0·56 ± 0·03 | 25 ± 2 |

TABLE 4. The effect of MTU on thyroid, skin and hair content of $^{131}I$ in male rats 9 days after injection of radio-iodide

(Six rats (280–320 g body wt.) in each group.)

| | Controls | MTU treated | *t* test of significance of difference (*P* values) |
|---|---|---|---|
| Thyroid $^{131}I$ (% injected dose) | 1·37 ± 0·11 | 0·12 ± 0·03 | < 0·001 |
| Skin $^{131}I$ (% injected dose) | 0·33 ± 0·03 | 0·84 ± 0·39 | N.S. |
| Hair $^{131}I$ (% injected dose) | 0·66 ± 0·08 | 1·27 ± 0·20 | < 0·05 |
| Total (skin + hair) $^{131}I$ content | 0·98 ± 0·08 | 2·15 ± 0·39 | < 0·05 |
| % dose/g hair | 0·15 ± 0·01 | 0·31 ± 0·03 | < 0·01 |
| % dose/g new-grown hair | 0·42 ± 0·13 | 0·40 ± 0·17 | N.S. |

there is no significant difference between animals with and without MTU in respect of skin values after thiocyanate (Group 2 compared with Group 4). The fact that no significant difference was observed between these two groups could mean that the irreversible uptake of $^{131}I$ was not due to organic binding iodine by a mechanism that can be inhibited by MTU. However, this $^{131}I$ fraction would be only a small proportion (5–10 %) of the total skin radioactivity and a difference of this order might well be obscured by individual variation in an experiment in which such a small number of animals is used.

The effects of chronic MTU administration on the uptake of $^{131}I$ by the hair was therefore examined. Twelve rats (280–320 g body wt.) were used, six controls and six animals receiving MTU 45 min before and again 5 hr

after the injection of 40 $\mu$c of $^{131}I$ and given MTU in the drinking water for 96 hr. The design of the experiment was otherwise identical to those involving KSCN and $KClO_4$ administration. The results show that MTU does not reduce but may even increase the uptake of $^{131}I$ by the hair. A second similar experiment on younger rats (180–200 g body wt.), in which the animals drank MTU solution for 6 days after $^{131}I$ injection, also gave results indicating a consistent, though in this case not statistically significant, increase in the $^{131}I$ content of both skin and hair (Table 5). The differences in thyroid $^{131}I$ content and thyroid weight show that the animals were receiving an effective amount of MTU.

TABLE 5. The effect of MTU on thyroid, skin and hair $^{131}I$ content 9 days after injection

(Five rats (180–200 g body wt.) in each group.)

| | Controls | MTU treated | $t$ test of significance of difference ($P$ values) |
|---|---|---|---|
| Thyroid wt. (mg/100 g initial body wt.) | 4·9 ± 0·2 | 9·3 ± 0·7 | < 0·001 |
| Thyroid $^{131}I$ (% injected dose) | 2·03 ± 0·20 | 0·10 ± 0·02 | < 0·001 |
| Skin $^{131}I$ (% injected dose) | 0·51 ± 0·14 | 0·71 ± 0·11 | N.S |
| Hair $^{131}I$ (% injected dose) | 0·52 ± 0·21 | 0·73 ± 0·12 | N.S |
| Total (skin + hair) $^{131}I$ (% dose) | 1·03 ± 0·21 | 1·64 ± 0·23 | N.S |
| % dose/g hair | 0·25 ± 0·05 | 0·35 ± 0·06 | N.S. |
| % dose/g new-grown hair | 0·77 ± 0·24 | 1·24 ± 0·22 | N.S. |

## DISCUSSION

Leblond (1954) drew attention to the uniquely high iodine content of the hair of the rat and made several very interesting observations. He showed that the iodine was not present as a result of contamination with urine but was derived from the dietary iodine. By the use of radio-iodine and chromatography he showed that the radioactivity present in the hair 5 days after injection of $^{131}I$ was in the form of inorganic iodide. The chemical state of the iodine in clipped hair was later confirmed by Bangham & Terry (1957). In further experiments Leblond showed that the hair iodine content of thyroidectomized rats was the same as that of normals, thus excluding the possibility that the iodine was reaching the hair as thyroid hormone which was then de-iodinated in the course of exercising its known stimulating action on hair growth. Our own interest in hair iodine arose out of studies of the iodide-concentrating mechanism of the skin of the rat (Brown-Grant & Pethes, 1959; Brown-Grant *et al.* 1960). It was observed that the 3–5 % of an injected dose of $^{131}I$ that was still present in the skin of the adult rat 24 hr after injection decreased only very slowly over the next 10–12 days. The fraction of the total radio-activity of skin plus hair that was found in the clipped hair gradually increased over this period. In addition, the observations already referred

to, that thiocyanate, even when given as early as 2 hr after the injection of radio-iodide, does not completely discharge the $^{131}I$ from the skin, indicates that a rapid and irreversible uptake by the hair, possibly involving organic binding of iodine, may be occurring.

The results of the present series of experiments have shown that pre-treatment with thiocyanate or perchlorate greatly reduces the uptake of $^{131}I$ by the hair. Whether these compounds are effective because of an effect on the level of $^{131}I$ in the skin as a whole or because of an action upon an iodide-concentrating mechanism in the hair follicle itself is not certain. The second alternative appears more likely, as the zone of high iodide concentration in the skin is in the lower layers of the epidermis away from the hair follicles and because localized areas of high $^{131}I$ concentration have been observed in the region of the hair follicles in the skin of the rat (Brown-Grant *et al.* 1960).

Thiocyanate injected 3 hr after $^{131}I$ administration does not reduce the level of hair $^{131}I$ to that seen in animals pre-treated with thiocyanate (Table 1) thus confirming the existence of a rapid, irreversible uptake of $^{131}I$ by the hair. The hypothesis that this may be due to organic binding of iodine such as occurs in the thyroid and the mammary gland is not supported by the results of experiments in which MTU was administered. No effect of MTU on the initial concentration of $^{131}I$ by the skin was observed, nor was there any detectable change in the proportion of the skin $^{131}I$ that could be discharged by thiocyanate (Table 3). In long-term experiments MTU actually increased the uptake of $^{131}I$ by the hair, though this increase was statistically significant only in the first of two such experiments. These latter observations are not thought to be relevant to the question of the metabolism of iodine by the hair follicle but simply to reflect the increased amount of $^{131}I$ available to the hair when uptake by the thyroid is blocked by MTU. Leblond (1954) reports that the $^{127}I$ content of the hair of thyroidectomized rats on an iodine deficient diet was actually higher than that of intact controls on the same diet. This observation may have a similar explanation.

If it is accepted that in the present experiments MTU reached the skin in adequate amounts and that it was unlikely to have been rapidly metabolized there, then the lack of effect on the $^{131}I$ content of the hair, together with the observation that the iodine of clipped or plucked hair is present as iodide, would appear to exclude the possibility that organic binding of iodine plays a significant part in the incorporation of iodide in the hair. The basis of the rapid irreversible uptake of $^{131}I$ by the hair may be simply a one-way transfer of material by the iodide-concentrating mechanism of the cells of the hair follicle to a 'compartment' where the iodide is no longer in equilibrium with the blood. It is of interest, however,

to note that the region of the skin where $^{131}I$ is concentrated is one where the initial stages of keratin formation are occurring and that a region of similar biochemical activity exists in the hair follicle (Rothman, 1954). Ryder (1958) has shown that it is in this pre-keratinization zone of the active follicle that the $^{35}S$-labelled cystine, that later appears in the hair itself, is concentrated as early as 6 min and up to 3 hr after injection. The interaction of iodine with the sulphydryl groups of proteins *in vitro* is well known (Hughes, 1957; Cunningham & Nuenke, 1959), but little is known of its role, if any, in iodine metabolism *in vivo*. The relative stability of the sulphenyl iodide groups in certain proteins (Fraenkel-Conrat, 1955) raises the possibility that interaction of iodine with the sulphur-containing amino acids which are present in large amounts in the proteins of these regions may be involved in the iodine metabolism of the epidermis and the hair follicles.

SUMMARY

1. Thiocyanate and perchlorate markedly reduce the uptake of $^{131}I$ by the hair of the rat.

2. When thiocyanate is given 3 hr after the injection of radio-iodide, the uptake is reduced but is significantly greater than in animals treated with KSCN before the injection of $^{131}I$. This indicates a rapid and irreversible uptake by the hair.

3. Methyl-thiouracil has no effect on the establishment of a skin : plasma concentration gradient for iodide.

4. Incorporation of $^{131}I$ into the hair is not reduced and may even be increased by methyl-thiouracil, owing possibly to more being available when the uptake of $^{131}I$ by the thyroid is blocked.

5. These findings are discussed in relation to earlier work on the iodine metabolism of the skin and the hair of the rat.

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# THE EFFECT OF CYTOCHROME C ON THE OXYGEN CONSUMPTION OF TISSUES OF NORMAL AND CO-POISONED ANIMALS

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Many reports have appeared recently on the successful use of cytochrome C in anoxia (Proger & Dekaneas, 1946*a*, *b*; Ammon & Fedtke, 1948; Klar, 1949; Biörk, 1951; Stelter, 1953; Feinen, 1955) but the mechanism of its action in carbon monoxide poisoning or in other anoxiae is still unknown. Spett & Spioch (1958), investigating cytochrome C activity in acute CO poisoning in rabbits, recorded a significant increase of $CO_2$ elimination and a relatively smaller increase of $O_2$ uptake. They found that cytochrome C inhibited the fall in the alkali reserve in acute CO poisoning.

It is generally known that CO does not block cytochrome C activity but that it blocks cytochrome oxidase and inhibits the respiratory system (Warburg, 1926). The respiratory inhibition observed *in vitro* by Warburg was at a much higher CO tension than is ever seen *in vivo*, which has led to objections being raised to his thesis that the respiratory inhibition by CO *in vivo* was due to blocking of the cellular respiratory system and of cytochrome oxidase in particular. These objections are supported by the fact that the affinity of CO for cytochrome oxidase is 2000 times less than its affinity for haemoglobin. Bänder & Kiese (1955) were, however, able to block the respiratory system in isolated mitochondria at relatively low CO tensions, such as may be seen *in vivo*.

Our object was therefore to compare the $O_2$ consumption in tissues from normal animals and from animals which had been lethally poisoned with CO, and to investigate the role of cytochrome C in these systems.

## METHODS

The animals used were guinea-pigs of various strains, both male and female. They weighed 200–400 g and were fed on a mixed diet. The animals were placed singly in a chamber (volume 144·8 l.) in which they were exposed to a CO concentration of 1% (v/v).

The animals showed varied individual sensitivities to CO and died within 1–5 hr. After death the animals were immediately dissected, and slices of brain, heart muscle and liver, weighing 150 mg each, were taken according to Warburg's method (as described by Hawk, Oser & Summerson, 1956). The slices were placed in Warburg flasks, containing 1·9 ml. of mammalian Ringer's solution, with air as the gas phase, and the $O_2$ consumption was measured during incubation for 1 hr at 38° C. To one series of flasks 0·1 ml. of cytochrome C (Cyto-Mack, H. Mack, Jllertissen, Bayern) solution was added; the other series served as controls.

The carbonmonoxyhaemoglobin concentration in the blood of the poisoned animals was determined by the iodometric method of Schmidt (1950).

In a second group of experiments normal animals were killed by decapitation and brain slices immediately prepared. The oxygen consumption of these slices was determined as above, cytochrome C being added to some of the flasks. The $O_2$ consumption of both groups of tissue slices was expressed as ml. $O_2$/g wet weight tissue/hr.

## RESULTS

The oxygen consumption of slices of brain from both normal and CO-poisoned guinea-pigs and of slices of liver and of heart muscle from poisoned animals, together with the effect of the addition of cytochrome C, are shown in Table 1. The $O_2$ consumption of the brain slices from poisoned animals was less than that of slices from normal ones. Similar experiments with slices of liver and heart muscle from normal animals showed that the

TABLE 1. The effect of addition of cytochrome C on the $O_2$ consumption of tissue slices from normal and CO-poisoned guinea-pigs. Slices of tissue (150 mg wet weight) incubated in mammalian Ringer's solution with and without added cytochrome C for 1 hr at 38° C, with air as the gas phase

| Tissue | No. of observations | $O_2$ consumption (ml./g wet wt./hr) Mean | S.E. | Added cytochrome C ($\mu$g/g wet wt.) |
|---|---|---|---|---|
| Normal brain | 41 | 1·24 | 0·022 | **0** |
| | 12 | 1·23 | 0·026 | **6·0** |
| | 24 | 1·22 | 0·030 | **12·0** |
| CO-poisoned brain | 55 | 1·0 | 0·024 | **0** |
| | 25 | 1·28 | 0·030 | **12·0** |
| | | $P < 0·001$ | — | — |
| CO-poisoned liver | 20 | 0·64 | 0·024 | 0 |
| | 28 | 0·84 | 0·029 | 12·0 |
| | | $P < 0·001$ | — | — |
| CO-poisoned heart muscle | 25 | 0·39 | 0·010 | 0 |
| | 28 | 0·61 | 0·015 | 12·0 |
| | | $P < 0·001$ | — | — |

values of the $O_2$ consumption of these tissues were reduced by 8·6 and 25·0% respectively by CO poisoning. Although the addition of cytochrome C did not increase the $O_2$ uptake of brain slices from normal animals, in each instance, it produced a highly significant increase in $O_2$ uptake ($P < 0·001$) of slices from poisoned guinea-pigs, when added at the level of 12 $\mu$g/g wet weight of tissue.

In Table 2 are shown the effects of various levels of added cytochrome C on the $O_2$ consumption of brain slices from CO-poisoned animals. The maximum increase in $O_2$ consumption was produced by 6·0 $\mu$g cytochrome C/g wet weight of brain, although the $O_2$ consumption was restored to normal by 3·0 $\mu$g cytochrome C/g wet weight of brain. Levels of 24·0 $\mu$g or more of cytochrome C/g wet weight of brain failed to increase the $O_2$ consumption of CO-poisoned brain slices.

A mean level of 0·140 ml. of CO/ml. blood was found in the poisoned animals. If the mean haemoglobin content of guinea-pig blood is taken as 13·0 g/100 ml., then over 80 % of the haemoglobin was saturated with CO in the poisoned animals.

TABLE 2. Oxygen consumption of brain slices from CO-poisoned guinea-pigs, incubated at 38° C, with air as the gas phase, in the presence of various amounts of added cytochrome C

| Added cytochrome C ($\mu$g/g wet wt.) | 0 | 1·5 | 3 | 6 | 12 | 24 | 50 |
|---|---|---|---|---|---|---|---|
| No. of observations | 55 | 21 | 22 | 44 | 25 | 4 | 11 |
| Mean $O_2$ consumption (ml./g wet wt./hr) | 1·0 | 0·96 | 1·22 | 1·50 | 1·28 | 1·11 | 1·07 |
| S.E. | 0·024 | 0·022 | 0·022 | 0·014 | 0·030 | 0·045 | 0·024 |
| $P$* | — | $> 0·05$ | $< 0·001$ | $< 0·001$ | $< 0·001$ | $> 0·05$ | $> 0·05$ |

* The value of $P$ refers to the difference between the results with various additions of cytochrome and those without added cytochrome.

## DISCUSSION

These findings show that the fall in $O_2$ consumption by tissue slices, produced by CO poisoning of the animals, can be reversed *in vitro* by the addition of cytochrome C. The optimum level of added cytochrome C for brain slices was 6·0 $\mu$g/g wet weight tissue, which may be compared with the finding of Niebrój, Spett & Spioch (1959) that a dose of 5·0 $\mu$g cytochrome C/g body weight prevented the fall in blood alkali reserve in CO-poisoned guinea-pigs.

The mechanism of the cytochrome C activity in increasing $O_2$ consumption in CO-poisoned tissues may be explained by supposing that it increases the cytochrome oxidase activity (Feinen, 1955; Lang & Feinen, 1951; Stelter, 1953), possibly by preventing the increase in the threshold level of oxygen tension necessary for the cytochrome oxidase to act. Such an increase in threshold oxygen tension was observed by Bänder & Kiese (1955) at tissue CO tensions of 0·5–2·0 mm Hg.

The increase in $O_2$ consumption of brain slices to levels above the normal, which can be produced by cytochrome C when added to CO-poisoned slices, may be analogous phenomenon to the $O_2$ debt accumulated after severe exercise. In both cases there is a disproportion between the $O_2$ requirement of the cells and the amount of $O_2$ which is being supplied.

Cytochrome C has the effect of reducing this disproportion in CO poisoning by increasing the availability of $O_2$; it is without effect on normal tissue slices because there is no lack of available $O_2$ for the cellular oxidation processes.

Lastly, the results presented here show that the reduction in $O_2$ consumption of tissue slices produced by CO poisoning of the animal is not due to irreversible changes in a proportion of the cells, since this reduction can be completely reversed by the addition of cytochrome C. The reduction in $O_2$ consumption must be due, therefore, to an inhibition of the cellular respiratory system.

## SUMMARY

1. The effect of adding cytochrome C on the $O_2$ consumption of brain, liver and heart muscle slices from guinea-pigs poisoned with CO has been investigated by a manometric method.

2. The results show that CO causes a decrease in the $O_2$ consumption of slices from these three organs and that cytochrome C can reverse this effect when added *in vitro*. The increase in $O_2$ consumption produced by cytochrome C in poisoned tissues was 28·0% for brain, 31·2% for liver and 56·4% for heart muscle; these results were all highly significant. In brain, normal $O_2$ consumption was restored by 3·0 $\mu$g cytochrome C/g wet weight of tissue, while a level of 6·0 $\mu$g increased it above normal. Thus this effect of CO poisoning is reversible.

3. Cytochrome C, when added *in vitro*, had no effect on the $O_2$ consumption of normal brain slices.

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# GRADED POTENTIALS OF BREAM RETINA

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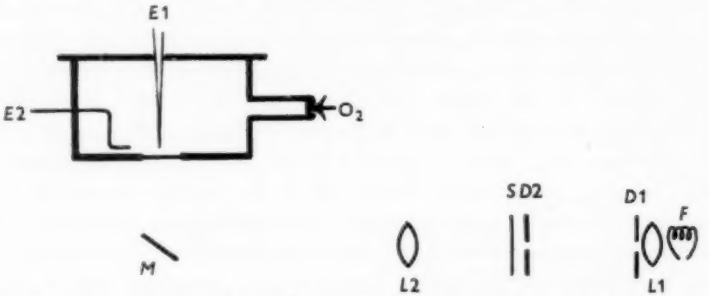
The large-amplitude graded potentials which can be obtained following illumination of fish retina were first reported by Svaetichin (1953). Although there is still evidence (Oikawa, Ogawa & Motokawa, 1959) to support the original hypothesis that they represent the activity of single cones, most subsequent studies have concluded that their source is located in more vitreal regions of the retina (Tomita, 1957; MacNichol & Svaetichin, 1958; Tomita, Murakami, Sato & Hashimoto, 1959). The amplitudes of these responses range from 10 to 30 mV and they are superimposed upon a negative d.c. potential of 10–50 mV. Two distinct types have been reported, a predominantly negative potential whose amplitude varies approximately linearly with the logarithm of the intensity of the stimulating light (Svaetichin, 1953) and a less common response which changes polarity with wave-length (Svaetichin & MacNichol, 1958; Motokawa, Oikawa & Tasaki, 1957).

This investigation is concerned with bream retina, which was originally reported to have the negative intraretinal potentials only (Svaetichin, 1953), but which will also be shown to exhibit responses that reverse polarity when the intensity of illumination is changed. Factors have been studied which would give an indication of the anatomical structure involved in the production of these graded intraretinal potentials, including the effects of light intensity, stimulus area, and electric current. Histological stains were employed in order to locate the position of the recording pipette and to examine retinal structures at this site. Spike activity in the immediate vicinity of the micro-electrode recording graded intraretinal potentials is described. Evidence is given to indicate that both the threshold and the effective stimulus area of the graded intraretinal potential is greater than what would be expected of the response of a single photoreceptor. Histological evidence strongly supports the hypothesis (MacNichol & Svaetichin, 1958) that the source of these potentials resides near the horizontal cell layer of fish retina. The electrical resistance of the structure across which these potentials are generated is relatively low and may be responsible for the ineffectiveness of current in altering the response.

* National Foundation Fellow.

## METHODS

*Preparation.* The results presented in this paper have been obtained from the excised retinas of bream (*Abramis brama*) but in the few studies *in vivo* of the e.r.g. anaesthetized animals were also used. Similar responses were obtained from the retinas of roach, trout, carp and tench but are not included in these results. The posterior hemisphere of the excised eye was cut in half and a strip of retina measuring about $10 \times 6$ mm at its widest margins was lifted away from the pigment epithelium, a manoeuvre facilitated by prior dark adaptation. The flat segment was mounted with its vitreal surface down on a thin cover-slip in the bottom of a Perspex chamber (Text-fig. 1), into which moistened oxygen was continuously pumped at a temperature of 18–22° C. The recording or stimulating micro-electrode was introduced through a circular hole in the movable upper section of the chamber while the secondary lead entered through a wax-sealed opening on its lateral side. The chamber was




Text-fig. 1. Apparatus: $D1$, variable stops imaged on retina by $L2$; $E1$, micro-electrode; $E2$, reference electrode; $F$, tungsten filament imaged at $S$ by $L1$; $L1$, condensing lens; $L2$, focusing lens; $M$, front-face mirror; $S$ shutter.

held in a mechanically driven micro-manipulator to facilitate electrode positioning and focusing of the stimulating light spot. The light entered through the bottom of the chamber and the vitreal surface of the retina, as in the intact eye. The entire preparation was viewed with a dissecting microscope at a magnification of $\times 40$.

*Optics.* The light source was a 6 V 36 W car head-lamp with a coiled tungsten filament. It was operated at 6 V d.c. and monitored by a voltmeter in parallel with the bulb. The filament was focused by lens $L1$ on a flag shutter $S$ where the beam could be readily interrupted (Text-fig. 1) by a hand operated electric relay. The lens $L1$ was focused on the preparation by lens $L2$, which was so positioned as to intercept the entire beam entering the diaphragm $D2$. Diaphragms of different diameters or neutral filters were interposed immediately in front of lens $L1$ to control either the area or intensity of illumination. The maximum illumination of the preparation measured by a Salford Electrical Instruments exposure photometer was approximately 21,000 lm/m$^2$.

*Electronics.* The most frequently used electrodes were 3 M-KCl single and double barrelled micropipettes with resistances of 5–20 MΩ. Pipettes filled with potassium ferricyanide and saline (0·9 % w/v) solutions were also employed. Electrochemically sharpened tungsten micro-electrodes, insulated up to 5–10 $\mu$ from the tip, were used routinely to record high-frequency impulse activity. The secondary junctions were chlorided silver wires which led to the customary cathode-follower input stage with grid current of $10^{-11}$ A. The chlorided silver wires were checked before experimentation for photo-electric effects. Direct coupling was used at all times for the study of low-frequency activity. The amplified signals were photographed from the tube face of a Cossor oscilloscope. Electric current was passed

through one barrel of a Y-shaped double-barrelled pipette, by means of a variable 122 V dry-cell battery in series with a 1000 MΩ Victoreen resistor, which was used to minimize current changes due to changes of the electrode's resistance. Most electrodes tolerate the passage of as much as 0·1 μA without marked changes in their resistances, but higher values proved more difficult, especially with electrodes having resistances greater than 5 MΩ. The most elusive property proved to be the coupling resistance, which varied not only from electrode to electrode, but also with the electrode's position in the tissue. The coupling resistances of most electrodes, as measured either in saline or on the surface of the retina, ranged from 50 to 200 kΩ but this value invariably increased markedly with subsequent insertion into the retina. Nevertheless, coupling resistances were never recorded high enough to reduce current passed through the electrodes by more than 50 % and usually a much smaller reduction occurred.

*Histology.* In order to locate the position of the recording pipette a staining technique identical to that recently reported by Tomita (1959) was used. Micropipettes were filled with a saturated solution of potassium ferricyanide and selected for resistances of 1–2 MΩ to provide large current-carrying capacity and at the same time to retain the ability to record the typical intraretinal graded potentials. The technique consisted in stopping the insertion of the micro-electrode immediately after recording the first graded potential, disconnecting the cathode-follower heater supply to prevent current leak, passing 1–2 μA for 1 min, quickly withdrawing the electrode and almost simultaneously dropping ferrous sulphate solution over the recording site. With the aid of the dissecting microscope, a small blue spot could be seen forming when current durations longer than 30 sec were used. To facilitate later sectioning the electrode tip was thereupon broken and the blunt end relowered to the surface of the retina in order to form a large marking spot. The size and density of the ferroferricyanide precipitate was dependent upon the time elapsing between the electrophoresis of the ferricyanide ion and the application of the ferrous salt. Only very faint spots were obtained when this duration exceeded 2 min. If the ferrous ions were applied before the electrode was withdrawn from the retina, however, the precipitate was formed principally at the electrode's tip and would be dislodged proximally upon withdrawal. The segments were fixed in Susa's reagent, imbedded in paraffin and sectioned at a thickness of 20 μ. These sections were stained with haematoxylin and eosin. Other sections of normal retina were studied with Azo carmine, iron haematoxylin and Van Gieson stains.

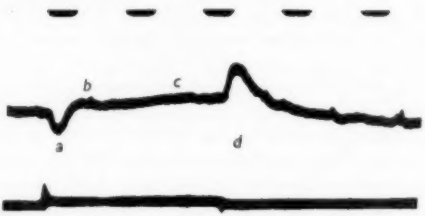
## RESULTS

### *Low-frequency response of bream retina*

If the conventional e.r.g. is recorded from the intact bream eye, the characteristic *a*-, *b*-, and *d*-waves are readily obtainable, as is shown in Text-fig. 2. The *b*-wave is largest after dark adaptation, as Day (1915) also reported to be the case in pike. A slowly developing positive *c*-wave can also be seen in Text-fig. 2. A few minutes after excision, however, the *b*-wave rapidly declines and the residual e.r.g. becomes diphasic, with a maintained negative wave at 'on' followed by a transient positive 'off' response. Such a change, presumably the result of deterioration, prompted the earliest retinal physiologists to study the amphibian retina, where similar changes are not seen for many hours after dissection (Day, 1915). If the excised fish eye is quickly opened and the extracted retina placed in an oxygenated moist chamber, the following pattern is observed. When the area of stimulation is less than approximately 3 mm in diameter a

micro-electrode on the photoreceptor surface will record a maintained positive wave during illumination and usually a transient 'off' response of opposite polarity. If either the area is increased, or the electrode is moved to an unilluminated region or if the retina itself is damaged, as, for example, after the electrode's insertion and withdrawal, the surface response will invariably become negative and can then reach amplitudes as large as 2 mV.

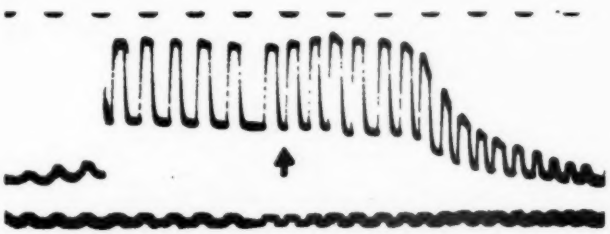
Accompanying the insertion of a micro-electrode into the retina from either the photoreceptor or vitreal surface there is an initial increase in its resistance of as much as 50 MΩ. If at this point the electrode is lowered further or if the table is gently tapped, the resistance is usually suddenly diminished and large graded photic potentials of 5–35 mV appear, super-




Text-fig. 2. E.r.g. *in vivo* after dark adaptation. Light stimulus begins at upward deflexion of lowest trace and ends at downward deflexion. Cornea positivity is upward on trace. Recording d.c. 'Off' response is 350 $\mu$V. Respiratory artifacts are visible. In all figures, time marker 1 sec.

imposed upon a negative d.c. potential of 5–50 mV. Only a negligible portion of this d.c. change is attributable to grid current. The over-all pattern of these events is ubiquitous across fish retina. With any given response there is a limited duration for which it can be maintained and within a few minutes there is always a reduction in the size of both the graded and d.c. potentials. The larger responses are more susceptible to this deterioration and often diminish markedly in a few minutes. Responses of 5–10 mV are readily obtainable with electrodes having tip diameters as large as 20 $\mu$, whereas the larger responses require fine micro-electrodes. Text-figure 3 shows a typical 25 mV negative response to an intermittent light appearing superimposed on a negative 12 mV d.c. potential upon lowering the electrode. At the arrow the electrode is gradually withdrawn and the response increases transiently. With further withdrawal the response diminishes together with the concomitant d.c. potential. The surface-negative response is now larger than the one elicitable before insertion. If the electrode is reinserted into the same area similar responses are reobtainable, but seldom are they as large. If the electrode is moved to a region only 200 $\mu$ away, however, larger responses are again obtainable.

If it is lowered deeper after a characteristic response is recorded, the response will gradually diminish together with its d.c. potential. Two or three such responses may be obtainable in a single traverse, as noted by Oikawa *et al.* (1959). Upon withdrawal, the same sequence of events may be reversed but the responses are invariably smaller. Such a pattern is also found when the micro-electrode is inserted from the vitreal side, but the responses are never as large as they are in the approach through the photoreceptors. The resistant hyalo-vitreal membrane of fish eye, upon which the retinal vessels course (Rochon-DuVigneaud, 1943), may be responsible for this difference. As a consequence, most results are based upon recordings obtained from the approach through the photoreceptors, and the vitreal approach was only used in later attempts to facilitate locating the site of the micro-electrode tip by iontophoresis.

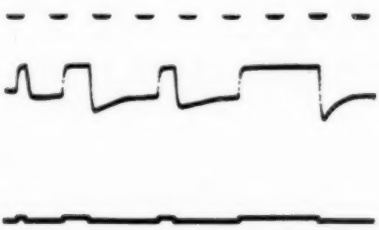


Text-fig. 3. Graded potential. Negativity upwards. Lowest trace, stimulus artifact; light 'on' when trace up. Maximum photic response 25 mV.

There are all gradations in the size of these potentials, from those that appear identical to the low-amplitude surface response to others as large as 35 mV. There are also differences in configuration, although they can be classified into two types representing extremes between which all intermediary responses are found. At one extreme is a predominantly negative response, which is the most frequent and the largest obtainable from bream retina (Text-fig. 3). At the other extreme is a response which at supramaximal levels of stimulation is also negative but at cessation of illumination exhibits a marked positive after-potential or 'off' response. This positive after-potential is very sensitive to the duration of illumination, being both larger and more accelerated with longer periods. A plot of response amplitude of this 'off' response against duration of illumination reveals an almost linear relationship up to periods of 5 sec, where a plateau is reached. Such a response is shown in Text-fig. 4, where the largest and most rapidly developing after-potentials are seen to follow the longest period of illumination. Svaetichin (1953) has also reported an acceleration in the rise and decay times of these intraretinal potentials, which occurred with light adaptation. The alternating stimuli of short and long duration

serve to demonstrate that this effect is not cumulative but immediate and transient. Such a dependency of 'off' responses on the duration of the preceding period of illumination has also been observed by Adrian & Matthews (1927) and Granit & Riddell (1934) in the amphibian e.r.g.

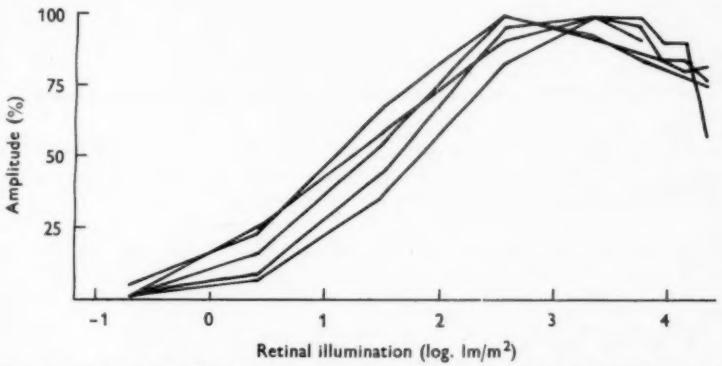
*The effect of intensity on response amplitude.* The amplitude of the intraretinal potentials of bream retina have been reported to vary linearly with the logarithm of the light intensity up to a saturation point at high intensities (Svaetichin, 1953). Absolute measurements on the level of retinal illumination, within which this linear relationship holds, are lacking. Although Mitarai & Yagasaki (1955) have reported increases in the d.c. potential, the effect of light adaptation has been found to be negligible (Svaetichin, 1953). It was considered important to determine the lowest



Text-fig. 4. Graded potential with 'off' response. Voltage calibration as Text-fig. 6. Negativity upwards. Lowest trace, stimulus artifact; light 'on' when trace up.

level of retinal illumination at which these responses are recordable, the level within which they vary linearly with the log. intensity and the point at which saturation occurs. During this study negative after potentials which were dependent upon the intensity of illumination were noted. These latter effects are not considered adaptational in that they curtail the maximal elicitable response rather than the sensitivity. The entire retinal segment was illuminated in order to obviate the marked dependency of these potentials on stimulus area (Tomita, Tosaka, Watanabe & Sato, 1958; Oikawa *et al.* 1959). The stimuli were flashes of 1–5 sec duration. Because of the diversity in response amplitude and configuration, as well as the inevitable deterioration that occurs with a prolonged study of an individual response, the following criteria were adopted in selecting data. Only predominantly negative potentials of greater than 15 mV with maximal stimulation were studied and responses which were not maintained almost constant to a submaximal stimulus throughout the test period were discarded. The latter criterion was the most difficult to attain because the test period lasted longer than 3 min, during which time there was frequently a diminution in the amplitude of the larger potentials.

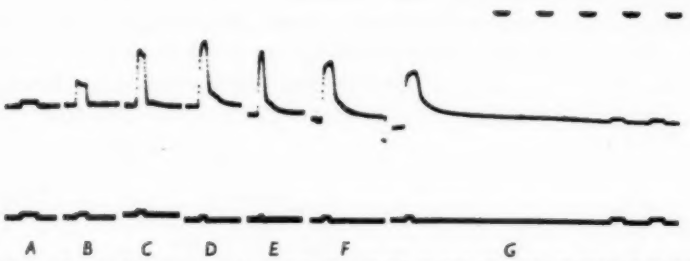
Text-figure 5 shows the result of this study where response amplitude is plotted as a percentage of the maximum response in order to minimize scatter. The responses are detectable only with difficulty below a retinal illumination of 0·1 lm/m², but vary almost linearly with the logarithm of the light intensity from 1 lm/m² up to retinal illuminations of 1000 lm/m², where saturation occurs. The decrease in response amplitude with high intensities represents the effect of both deterioration and the superposition of responses on a lingering after-potential. Text-figure 6 demonstrates a set of typically negative responses to increasing intensities of illumination. After-potentials characterized by an elevation of the negative d.c. potential occur minutes after intense 1–2 sec flashes. If threshold stimuli are given



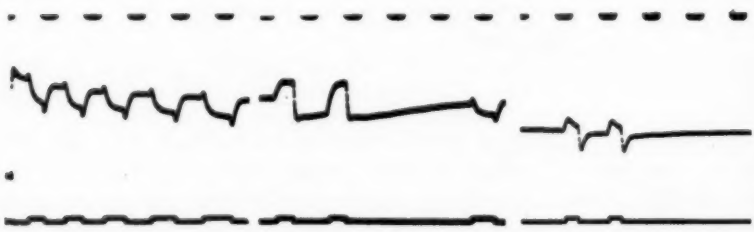

Text-fig. 5. Graph of amplitude of graded negative potentials plotted against retinal illumination. Semi-log. scale.

during a negative after-potential, the amplitudes of the threshold responses are unchanged, indicating that there is no change in sensitivity. In addition to the negative after-potential, another change can be seen to occur with increasing intensities of stimulation. At 35 lm/m² there is an initial transient at the beginning of stimulation. Such a transient is also apparent at 350 lm/m², but is lost at higher levels of illumination. This is shown in its most exaggerated form in the case of responses with large positive after-potentials demonstrated by Text-fig. 7. Stimulation with 3 lm/m² (*A*) produces a maintained positive potential but with an initial negative transient at the beginning of illumination, as in the case of the response of Text-fig. 6. With 350 lm/m² (*C*) the response becomes negative during illumination but also has an initial transient. At cessation of stimulation there is a marked positive 'off' response. With 1900 lm/m² (*B*) the initial negative transient disappears and the response is almost a square negative wave during illumination and a rapidly developing

positive after-potential at 'off'. If a second stimulus is delivered during the course of the positive after-potential the amplitude of the negative wave is correspondingly greater. It can be seen that despite the thousand-fold increase in stimulus intensity the amplitude of the positive after-potential is fixed, although its duration varies considerably. There appears to be a saturation point that is rapidly reached at both the positive and negative levels of these intraretinal responses, so that any additional




Text-fig. 6. Graded response to *A* 0·2, *B* 3, *C* 35, *D* 350, *E* 1900, *F* 5300, *G* 21,000 and 0·2 lm/m² terminally. +10 mV calibration at *F*. Negativity upwards. Lowest trace, stimulus artifact; light 'on' when trace up.

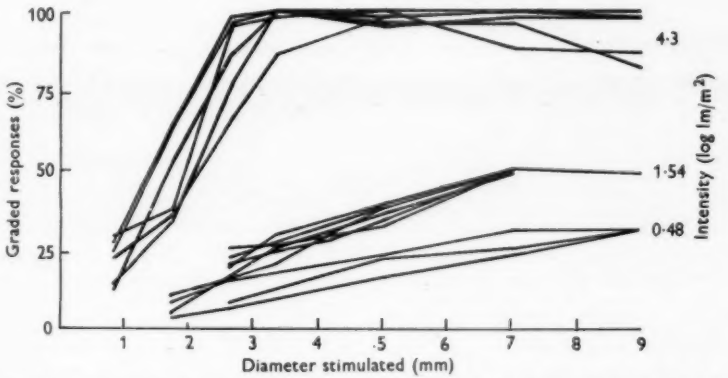



Text-fig. 7. Graded response. *A* 3, *B* 1900 and terminal 3 lm/m², *C* 350 lm/m². d.c. potential at beginning of *A* trace, reduced at *C*. Voltage calibration as Text-fig. 6. Negativity upward. Lowest trace, stimulus artifact; light 'on' when trace up.

increase in stimulus intensity serves only either to prolong the duration or to accelerate the rise or fall times of the response. The initial negative transient and the prominent 'off' response may indicate that the positive component of this response develops and decays more slowly than the negative component, in a manner similar to the mechanism proposed for the positive component of the colour response of *Mugil* retina (MacNichol & Svaetichin, 1958). The delay in the development of this component may also explain the noticeable dependency of the positive after-potential on the duration of illumination.

*The effect of area on response amplitude.* Two previous studies (Tomita

*et al.* 1958; Oikawa *et al.* 1959) have reported the marked dependency of these intraretinal potentials on the area of stimulation. The earlier workers, employing an illumination of about 200 lm/m$^2$, noted that they became very small when the diameter of the light spot was 0·2 mm, and increased in amplitude progressively to diameters of 3·2 mm. The subsequent workers, although not stipulating light intensity, found two different patterns with increasing areas of stimulation. The most common was similar to that which Tomita *et al.* (1958) described, in which the response amplitude increased up to diameters of 1·5–2·0 mm. A second, less common pattern, obtainable only with electrodes having tips finer than 0·2$\mu$ in

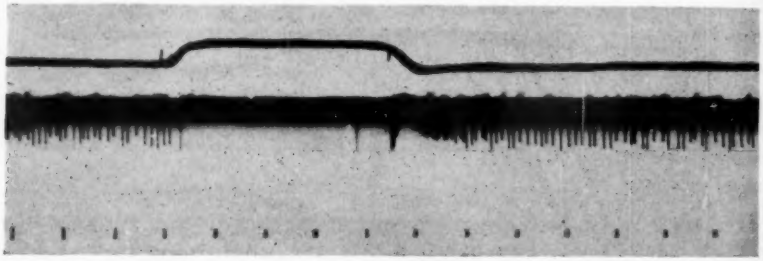



Text-fig. 8. Graph of amplitude of graded responses plotted against diameter of retina stimulated with supramaximal, submaximal and threshold illumination.

diameter, showed no increase in response amplitude with diameters greater than 0·16 mm, although changes in the after-potentials were observed. As a result of this and additional histological evidence Oikawa *et al.* (1959) attributed the latter responses to cones.

In this study the effect of area was also investigated but with more attention being given to the intensity of stimulation. The criteria for selecting responses were identical to those of the previous section. Stimulating spots of gradually increasing diameters were focused on the retina around the micro-electrode, and the effective area for a response was defined as the area beyond which illumination produced no increase in response amplitude. Text-figure 8 shows the results with amplitude again being plotted as percentage of the maximum response. Despite supramaximal stimulation, a retinal area of greater than 2 mm in diameter has to be illuminated in order to obtain a maximal response. This value must be a lower limit of the effective area of the structure generating the intraretinal response. Complete elimination of non-focal stray light can only

increase this value and focal stray light can be considered inconsequential. On the other hand, with stimuli only slightly above threshold the effective area is found to include the entire retinal segment. In this case focal stray light may be a contributing factor in increasing the limit, whereas non-focal stray light can be disregarded. Nevertheless, the area influencing the generator of the graded intraretinal potentials must be considered relatively large, having a lower limit diameter of 2 mm and an upper one of at least 8–10 mm. The small receptive fields described by Oikawa *et al.* (1959) were not encountered. The fact that these investigators did find changes in the after-potentials with larger areas may indicate that they



Text-fig. 9. Graded response and adjacent spike activity. Graded response 7 mV and spike amplitude 50 $\mu$V. Negativity is upward for upper trace, downward for lower. Upward artifact on upper trace indicates light 'on' and downward light 'off'.

were operating in the plateau of the intensity–amplitude curve, above retinal illuminations of 1000 lm/m$^2$.

There is a marked similarity between this result and that obtained by Wirth & Zetterström (1954) in their study of the effect of area on the size of the cat's e.r.g. In order to obtain an e.r.g. having the characteristic features of the high-intensity response it was necessary to stimulate the retina with diameters of 3–5 mm, and with stimuli less than 2 mm in diameter no e.r.g. could be recorded.

### *High-frequency unit activity*

'Spikes' are easily recorded with insulated tungsten micro-electrodes from either the vitreal or photoreceptor surfaces of bream retina. The responses in general resemble those of ganglion cells of other vertebrate retinae (Hartline, 1938; Granit & Svaetichin, 1939). One noteworthy characteristic encountered in the study of discharges at the vitreal surface is their frequent sensitivity to the pressure of the recording electrode. Gradual and continued pressure can produce discharge rates of 200–300/sec in a reversible manner similar to what Alanis & Matthews (1952) described

of central neurones. The activity recorded at the photoreceptor surface is of more interest, however, in that it would appear to represent propagated responses of layers more external than the ganglion cells. Although spikes are obtained at the photoreceptor surface, they are always increased in amplitude by inserting the electrode into a retinal region within or just beyond which the large intraretinal potentials are recorded. With stimuli greater than 2000 lm/m$^2$ the 'spikes' at this location are invariably 'off' units, which always maintained a spontaneous rhythmic discharge in the dark. Simultaneous recordings of both the graded intraretinal potentials and the neighbouring spike activity are shown in Text-fig. 9. At this level of retinal illumination the graded potentials are all negative during the period of illumination, as described previously, and respond at 'off' with varying after-potentials. Spike activity always decreases during illumination and increases at 'off' in direct relationship to the positive after-potentials of the graded response. Single units can be maintained relatively constant as long as 2 hr after dissection. With prolonged study, however, a change occurs in the discharge, initially noticed as an increased frequency of firing in the dark. Illumination is only able to curtail the discharge momentarily, after which there is inevitable escape. Eventually the unit disappears in a high-frequency discharge in the dark and can only be made to fire during illumination. This rapid increase in frequency with subsequent cessation of activity is similar to depolarization or cathodal block described of other nerve cells (Eccles, 1957), but although temporarily offset by illumination, is irreversible in the case of these cells. The amplitudes of the 'spikes' are inversely related to the frequency of firing, being smallest during the brisk 'off' response as seen in Text-fig. 9, and largest when firing slowly.

*Location of the micropipette and histology of bream retina*

In order to determine the intraretinal region from which the graded potentials are best obtainable, the Turnbull's blue-staining technique described above was used (Tomita *et al.* 1959). The earliest attempts revealed that the external nuclear layer of the retina has to be penetrated before the large graded responses are obtainable (Pl. IA). Subsequent stains produced by electrodes introduced from the more difficult vitreal approach were localized in the horizontal cell layer, without any evidence of disruption or dye in the region of the outer or inner limbs of the photoreceptors or in the external nuclear layer, confirming the observations that these layers are not the source of the large graded potentials (Pl. IB). Reduction in both current intensity and duration of iontophoresis produced the smallest stain obtainable in this study (Pl. IC) which is less than 30 $\mu$ at its widest diameter and is located almost entirely at the horizontal

cell layer of the retina. Six successive attempts with this technique were localized in this region of the retina.

The prominence of this cell layer in fishes was observed by early retinal histologists (Schiefferdecker, 1886). Although working predominantly with information obtained from macerated material Schiefferdecker compiled the comparative anatomy of the vertebrate horizontal cell layer. In fishes individual cells are flat and stellate in appearance with processes as long as 500 $\mu$. The cells are reported to form three distinct and almost continuous layers beneath the external plexiform layer. Mammals are second only to fishes in the prominence of this retinal layer, which is least developed in amphibians. The existence of intercellular anastomoses, observed by most early histologists, has been subsequently refuted by Polyak (1941), but only recently resupported by Chalissery (1955).

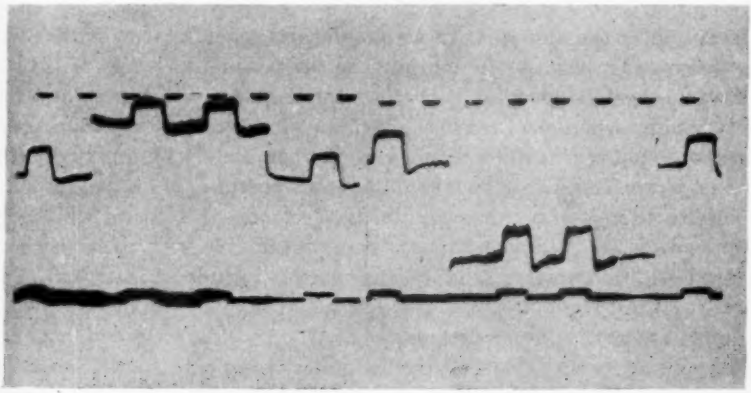
The present histological studies show that the horizontal cell layer of bream retina forms an almost continous sheet of closely packed cells extending immediately beneath the external plexiform layer. The diameter of the somata of the largest cells is 40 $\mu$ if a 20% shrinkage factor is assumed. Plate II B shows a tangential section through the somata of these cells, revealing the layer as the micro-electrode 'sees' it. Between their hexagonal somata are oval extracellular spaces through which the fibres of both Müller and bipolar cells have been reported to course, the former to form the external plexiform layer. The large processes of the horizontal cells extend from their bipolar surface to run both horizontally and vertically interdigitating in a dense network at the outer surface of the bipolar cell layer. Plate II A reveals such a single process extending 150 $\mu$ from its soma, which is visible in the same section. It can also be seen that in any one vertical section the somata of the horizontal cells overlap, forming what may have been interpreted as more than one layer by investigators using macerated preparations. With these staining techniques it was never possible to discern intercellular anastomoses between adjacent horizontal cells.

### *The effect of electric current*

If these graded intraretinal potentials represent the transmembrane potentials of single cells it would be expected from analogy with receptor and post-synaptic potentials (Eccles, 1957) of more extensively studied nerve cells that alterations in the d.c. potential would produce alterations in response amplitude. MacNichol & Svaetichin (1958) have already reported that $10^{-8}$ A passed through the recording barrel of a micropipette failed to effect the response. If the response is intracellular, then either the membrane potential was not changed sufficiently by the current because of the cell's low total resistance, or the generator of these potentials

is an active ionic movement independent of membrane potential, somewhat analogous to what Lundberg (1956) reported of salivary-gland cells. If the recorded response is extracellular the explanation is simplified, because the current would then produce little change across the active membrane.

These possibilities can be investigated by the use of double-barrelled electrodes, which allow membrane potential to be measured simultaneously with the passage of current. The most difficult problem associated with this measurement proves to be the coupling resistance between the barrels of the electrode. The coupling resistance is lowest at the surface of the retina (25–200 kΩ) and largest during insertion (up to 100 MΩ). The most



Text-fig. 10. Graded potentials while $1{\cdot}2 \times 10^{-7}$ A being passed through one barrel of double-barrelled pipette. Two different responses: left, hyperpolarization and decrease in negative response; right, depolarization and increase in negative response. Voltage calibration as Text-fig. 6. Negativity upwards. Lowest trace stimulus artifact; light 'on' when trace up.

typical sequence is to record an enormous increase in coupling resistance just before recording the d.c. potential and the large graded responses. At this point the coupling resistance suddenly drops to approximate to the surface value. To obtain the total resistance of the hypothetical cell, the coupling resistance as measured at the surface of the retina is subtracted from the resistance measured during the recording of the large graded response. The resistances so calculated range from 10 to 200 kΩ and are obtained with currents as great as $10^{-7}$ A. Because of these relatively low values, current of $10^{-7}$ A is unable to alter the resting potential by more than 10–20 mV. In two cases, however, the response was altered with maximum current intensity. Text-figure 10 shows a

reduction of 1·5 mV in the amplitude of a negative response produced by inward current of $10^{-7}$ A. In a second case outward current increased the amplitude of a similar response by 0·5 mV. This alteration of response amplitude is in the direction that would be expected for intracellular responses resulting from permeability changes of a cell membrane. It may be equally explicable, however, by a decrease in resistance due to illumination of a structure across which the current is passing. A decrease in the radial resistance of frog retina has been proposed to explain changes in the amplitude of the *b*- and *d*-waves of the e.r.g. with polarizing currents (Brindley, 1956) and a decrease in the resistance of goldfish retina during illumination has recently been found (Tasaki, 1960).

## DISCUSSION

In favour of the view that these intraretinal potentials are intracellular are the resting negative potential, the large amplitude of the graded potential, the tendency for both to deteriorate concomitantly and relatively rapidly when recorded with even fine pipettes, and the individuality of response pattern at different regions of a completely illuminated retina. Against it are Tomita's (1957) simultaneous recording of graded responses from both barrels of a concentric micro-electrode, the tips of which were separated by 50 $\mu$, the fact that they are obtainable with relatively large electrodes (20 $\mu$ diameter), that receptive fields include at least half of the retina, that intracellular spikes are absent and that the response amplitude tolerates extremely large currents.

Measurements with double-barrelled pipettes indicate that the resistance between the recording electrodes is 10–200 kΩ greater than the surface measurement when the recording pipette is in that region of the retina where the large graded potentials are found. This increase in resistance can be attributed to either the micro-electrode's mechanical alteration of the retinal tissue, or its encroachment upon a high resistance structure, or its entrance into an electrically isolated intraretinal compartment. The latter hypothesis is supported by the following facts. The change in resistance occurs concomitantly with the appearance of a steady negative potential of 5–50 mV, which is independent of the position of the external reference electrode; that is, the d.c. potential exists when the retinal segment is completely submerged in a conducting solution. This negative potential, the large graded responses, and the increased interelectrode resistance occur suddenly and after only a slight movement of the recording pipette, observations which seem more explicable by a compartment hypothesis. There is a gradual and greater increase of interelectrode resistance just before the appearance of the above phenomenon, which seems more attributable to either mechanical changes or the pipette's

approaching a high-resistance structure. This change occurs at the region of the photoreceptor nuclei as indicated by the marking techniques and may be due to a structure similar to the R membrane reported to be present in frog, rat and rabbit retina (Brindley, 1958).

Iontophoretic stains locate this hypothetical compartment at the external plexiform or horizontal cell layers. Histology reveals that the horizontal cells of fish retina are relatively large and also form a compact tangential structure shunted only by cylindrical extracellular spaces which form 10–20 % of any cross-sectional area (Pl. II). These spaces are in addition filled with both nerve and Müller fibres that would act to increase the radial resistance of this layer. That the graded responses are most readily found in fish retina where the horizontal cell layer is most prominent, and are almost absent in amphibian retina where it is least developed (Shiefferdecker, 1886), suggests that this layer plays a part in the genesis of the large graded potentials. Similar responses have also been detected, although with less facility, in mammalian retinae (Grüsser, 1957; Brown & Wiesel, 1958), which are second to those of fish in the prominence of the horizontal cell layer (Schiefferdecker, 1886).

The compartment may be the intracellular space of a horizontal cell, although its specific membrane resistance would have to be extremely low if its surface area were even as large as a motoneurone (Eccles, 1957) to have a total resistance as low as 10 kΩ. If the syncytial hypothesis is true, then the lower resistance would be expected, although the demonstrations of these anastomoses extant in the literature only show processes having the dimensions of fine dendrites (Chalissery, 1955). Another hypothesis is offered which would envision the external nuclear layer or the external limiting membrane and the compact horizontal cell layer of fish retina forming one or a number of relatively large isolated compartments of the external plexiform layer and this would explain most of the results of this investigation more satisfactorily.

There are many similarities between the graded responses and the lower amplitude e.r.g. Both require large areas of illumination, both have relatively high thresholds, both have 'off' responses which are very dependent upon the duration of illumination and both have fast initial negative components. In addition, changes in amplitude of both these responses require rather large currents and the direction of these changes can be explained by a decrease in resistance during illumination of the structure across which the current is passing. In general, there is only a small d.c. component in the vertebrate e.r.g. during illumination as demonstrated *in vivo* by the fish e.r.g. of Text-fig. 2. The surface response of isolated fish retina, however, frequently shows a pronounced d.c. change during illumination, similar to that of the graded intraretinal response.

The vertebrate e.r.g. appears to be generated across a high-resistance tangential structure in the retina, which has been identified as the external limiting membrane and across which current is most likely carried by the photoreceptors (Brindley, 1958). If the potentials resulting from this current are generated across the high-resistance boundary on the photoreceptor side of the hypothetical compartment, this current could also be the source of the graded intraretinal potentials, and a micro-electrode located within the compartment would be expected to record these potentials at a greater amplitude than it would at the retinal surface.

In addition to the e.r.g., focal intraretinal potentials have occasionally been reported to occur in amphibian retina (Tomita & Torihama, 1956). These potentials differ from the e.r.g. in their greater susceptibility to deterioration and their complex changes to stimulation (Brindley, 1958). Such complexities are reminiscent of the polarity reversals found in the graded intraretinal responses of fish retina. These potentials may be a limited version of the phenomenon proposed to be occurring in fish retina, since they are less frequently detected, deteriorate quickly, and are of a much smaller amplitude. The fact that they are not as well maintained during illumination as the graded intraretinal potentials of fish may reflect their relationship to the amphibian e.r.g., which is as dissimilar from the surface response of excised fish retina.

The presence of extracellular spikes both at the photoreceptor surface and within the region from which large graded responses are recorded suggests that their source is more external than the ganglion-cell layer. There is a close relationship between the graded potentials and neighbouring spikes, with positive graded potentials being associated with increased frequency and decreased amplitude of the spikes and the reverse relationship for the negative potentials. Such a correlation between spikes and graded potentials would not be expected if these activities originated in separate cells. It is also in agreement with the conventional intracellular changes at receptor and dendritic terminals with increased positivity being associated with depolarization and increased frequency of propagated action potentials, and the reverse with negativity. This association may indicate that the graded fish potentials represent the activity of bipolar processes, isolated as they are within the external plexiform layer by the horizontal cells, and the spikes the propagated responses of the bipolar somata occurring outside this hypothetical compartment and hence of smaller amplitude.

The fact that two processes of opposite polarity appear to be acting in varying degrees in the graded potentials of bream retina indicate that the negative response does not merely sum luminosity. At low intensities the positive potential is most conspicuous, but may remain observable in some

responses even at the highest intensities, as an after-potential or 'off' response. At illuminations greater than 350 lm/m$^2$ the responses are always negative during illumination and are associated with curtailment of neighbouring spike activity. The fact that some responses change polarity with intensity make them seem analogous to the wave-length reversing potentials of other fish retina, although colour-discriminating responses have been reported to be absent from bream retina (Svaetichin, 1953). This polarity reversal is not due to partial activation of a tangential structure, since it occurs with illumination of the entire retina. The author has observed polarity reversals with colour while studying bream retina, but they were never properly controlled for changes in intensity. The fact that intensity does play a part in producing polarity reversal demands that a careful distinction be made between intensity and wave-length in any spectral scanning study. Although two processes of opposite polarity and different spectral sensitivities are summing in the same response in the case of *Mugil* retina (MacNichol & Svaetichin, 1958), since multiphasic patterns were not obtained in a spectral scan it would be interesting to know whether these two processes also differ in their threshold intensity. The complexity of such a system would prevent a straightforward comparison of spectral sensitivity curves of intraretinal potentials with those of photochemical pigments or with those obtained at a different intensity level and would perhaps explain some of the variation in such findings at the present time.

The role of these responses in fish vision is undoubtedly of major importance. They are either responses of the terminals of photoreceptors in the external plexiform layer or adjacent second-order neurones consisting of either horizontal cells or the dendrites of bipolars. That two processes of different threshold and temporal relationships occur suggests that there are either at least two types of photoreceptor responses, or two types of transmitter substances released at this stage in the visual pathway which sum in different degrees to produce the graded intraretinal potential.

SUMMARY

1. Evidence is given to support the hypothesis that the graded intraretinal potentials of bream retina originate near the horizontal cell layer of the retina.

2. Two components of opposite polarity and different temporal sequence sum to varying degrees in the graded intraretinal response. With total retinal illumination greater than 350 lm/m$^2$ the graded potentials are negative, but at lower intensities slower developing maintained positive potentials are found which are manifest only as 'off' responses at higher intensities.

3. The graded negative intraretinal response is recorded with difficulty below a retinal illumination of 0·1 lm/m²; from this point it varies almost linearily with the logarithm of illumination to 1000 lm/m². Greater illumination produces increases in the duration of the after-potentials but no increase in amplitude.

4. The minimum area of retina that must be illuminated to produce maximum response with supramaximal stimuli is 2 mm in diameter and summation occurs over areas as large as 8 mm in diameter.

5. The resistance of the structure across which these potentials are generated ranges from 10 to 200 kΩ and supports the hypothesis that these potentials arise in extracellular intraretinal spaces.

I wish to express my sincere thanks to Dr W. A. H. Rushton, Mr Clive Hood and Mr Canwell for their indispensable assistance.

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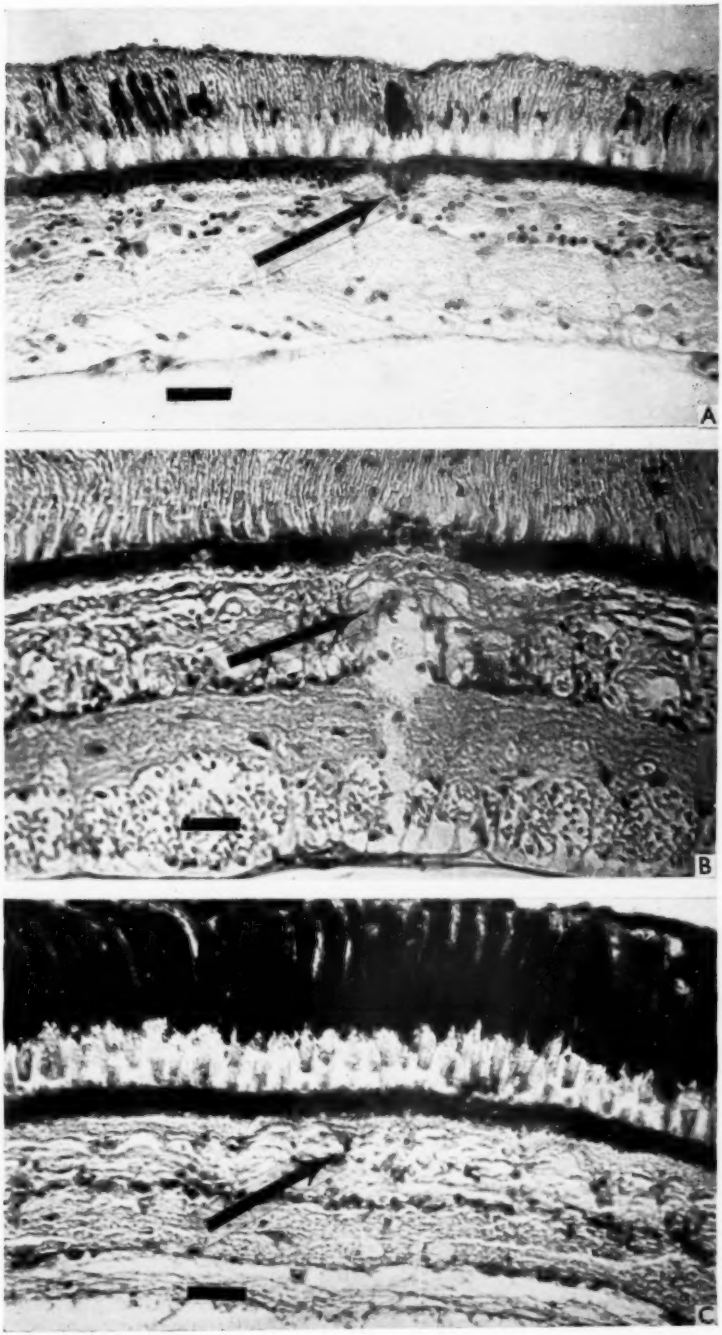
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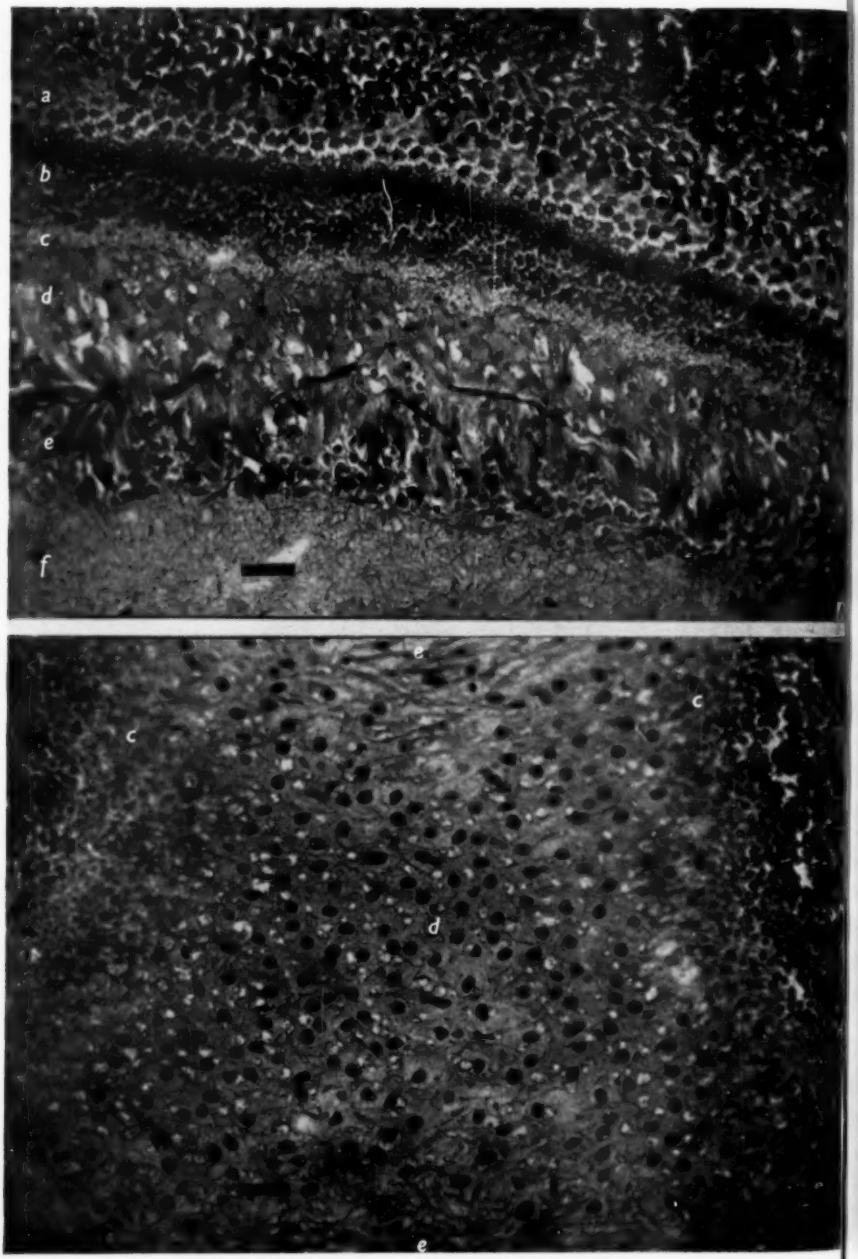


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## EXPLANATION OF PLATES

### PLATE 1

Location of distinctly blue ferroferricyanide precipitates intraretinally. A and C via photoreceptors; B via vitreal approach. Lines represent 40 $\mu$.

### PLATE 2

A, radial section of bream retina; B, tangential section. *a*, layer of rods and cones *b*, layer of photoreceptor nuclei, *c*, external plexiform layer, *d*, horizontal cell layer, *e*, bipolar cell layer, *f*, internal plexiform layer. Lines represent 40 $\mu$.

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# CHANGES IN CARDIAC AND RESPIRATORY FUNCTION, AND IN BLOOD CARBON DIOXIDE PRESSURE AND pH, IN CATS EXPOSED TO OXYGEN UNDER HIGH PRESSURE

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Earlier experiments on rats (Taylor, 1958) showed that on exposure to pure oxygen under a pressure of 6 atmospheres, slowing of the heart and of respiration took place much earlier when the rats were anaesthetized with chloralose than when barbiturate was the anaesthetic. There was evidence, too, that such slowing was accompanied by a fall in pH of the arterial blood, but there was no evidence of a rise in $CO_2$ pressure before the onset of cardiac or respiratory failure.

These experiments suffered from the disadvantages that the rats underwent a fairly rapid and considerable fall in body temperature, that the size and frequency of blood samples were strictly limited and that only arterial blood was sampled. It was desirable therefore to repeat and extend the work on larger animals such as cats.

## METHODS

Young cats of both sexes (av. wt. 1·50 kg) were used in all experiments and were anaesthetized by an intraperitoneal injection of either an aqueous solution of 1 % chloralose and 10 % urethane (1 ml./100 g body wt.) or with pentobarbitone sodium B.P. (Veterinary Nembutal; Abbott Laboratories Ltd.; 50 mg/100 g body wt.).

Any such procedures as vagotomy and section of sinus nerves were then carried out before heparin dissolved in 0·9 % NaCl solution (5000 i.u./ml.) was injected intravenously (0·5 ml./kg body wt.). The femoral arteries were then cannulated with polythene tubing, as was the right atrium via the right jugular vein, the animal was placed in the pressure chamber, and the appropriate connexions made to recording and sampling apparatus. The method of compression was as has already been described (Taylor, 1956).

Heart rate, mean arterial blood pressure, respiration, body temperature and blood pH were recorded as described by Taylor (1958). In the first seven experiments the recorded pH was that of the femoral arterial blood; thereafter, it was that of the mixed venous blood drawn from the right atrium.

Blood samples were collected and analysed for total $CO_2$ and $O_2$. The $CO_2$ pressure was calculated as in the earlier experiments, using the form of the Henderson–Hasselbalch equation and the tables given by Severinghaus, Stupfel & Bradley (1956*a*, *b*). A series of both arterial and right atrial samples could be collected in any experiment, but in any one experiment the pH could be recorded, and thus the $CO_2$ pressure calculated, for one type of sample only. A rapid flow of blood through the chamber containing the glass electrode permitted the pH to be recorded at a blood temperature not significantly different from

body temperature, but in some cases the pH was recorded at a fixed time after sampling and the appropriate temperature corrections calculated by Rosenthal's formula (Rosenthal, 1948), the temperatures being checked by a fine thermocouple fitted into the chamber containing the glass electrode.

## RESULTS

As in the rat experiments, abnormalities of the electrocardiogram were frequently observed. These included inverted or absent P waves, increase in P-R interval, changes in the QRS complex, and inverted or biphasic T waves. Unlike the rats, the cats showed no evidence of lung damage, other than mild hyperaemia. And, again in contrast with the rats, convulsions were observed frequently in cats anaesthetized with chloralose and urethane, and rarely when barbiturate anaesthesia was employed.

*Comparison of changes in cats anaesthetized with chloralose and urethane and with Nembutal.* Chloralose and urethane were used to anaesthetize sixteen animals (group *A*) and nembutal for fifteen (group *B*), in each case without operative interference other than that required to permit blood sampling. In the first 7 experiments (4 from group A and 3 from group *B*) only femoral arterial blood samples were taken; in 3 experiments in each group, samples were taken only from the right heart, and in the remainder (9 from each group) samples were taken from both arterial and mixed venous blood.

*Convulsions.* Comparison of the two groups showed that convulsive movements, starting about 30 min after exposure to $O_2$ had commenced, occurred in thirteen out of sixteen animals in group *A* but only in two out of fifteen animals in group *B*.

*Respiration.* Obvious hyperpnoea was noted in one cat only in group *A* but occurred in thirteen out of fifteen cats in group *B*. Slowing and eventual failure of the respiration occurred in less than 2 hr in ten out of sixteen animals in group *A*, whereas in group *B* only two animals showed eventually an appreciable degree of respiratory slowing.

*Heart rate.* In all animals in group *A* the initial effect, 20–40 min after exposure to $O_2$ commenced, was a slowing of the heart rate, which in eight cats decreased to 50 % or less of the initial rate within 2 hr, and in only three instances did not decrease by at least one third. In group *B*, however, the initial effect was in every case a slight but distinct rise in heart rate. In no case did the heart rate fall by 50 % within 2 hr and in ten out of the fifteen animals the heart rate did not decrease even by one third in 180–240 min. Thus animals in group *B* survived for a much longer period, on the average, than did those in group *A* and, in fact, a number of experiments in group *B* were terminated simply on account of the passage of time and not because of any apparent deterioration in the condition of the animal.

*Hydrogen ion concentration.* Since samples of blood were not taken at the same times in all experiments, a strict statistical comparison of pH, total $CO_2$ and $O_2$ was not possible. Nevertheless, in respect of the twelve cats in each group from which samples of mixed venous blood were taken, it was found that blood pH tended to be lower in group *A* than in group *B*, falling to less than 7·20 in seven cases in group *A*. In group *B* this occurred only in three cases, and it was more usual for the pH to rise early in the experiment to a plateau and later to fall to near its initial level.

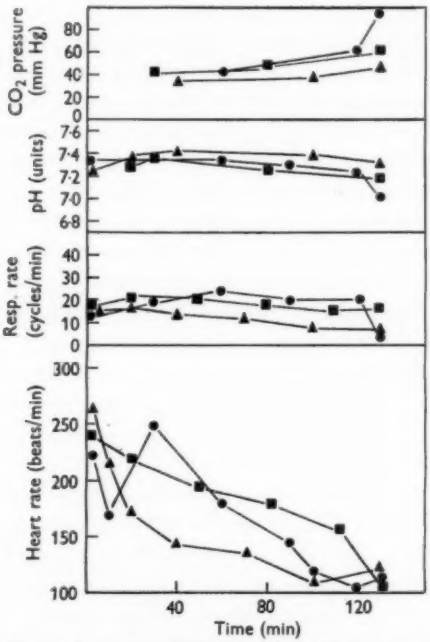


**Fig. 1.** Graphs (from above down) of calculated $CO_2$ pressure and pH of mixed venous blood from right atrium, respiratory rate and heart rate plotted against time from beginning of compression, for three consecutive experiments on cats anaesthetized with chloralose and urethane and exposed to $O_2$ under a pressure of 6 atm. ■, cat 14; ▲, cat 16; ●, cat 18.

*Carbon dioxide pressure of blood.* Not unnaturally, the calculated blood $CO_2$ pressure bore a close and inverse relationship to the measured pH. It was on the average a little higher in the earlier stages of experiments in group *A* than in group *B*; but even in group *A* large increases in $CO_2$ were not found except when obvious respiratory failure had supervened. Of the animals, twelve in each group, from which mixed venous blood samples were taken, in 7 experiments in group *A* the $CO_2$ pressure remained below

50 mm Hg in the presence of definite respiratory slowing and, in 4 instances, of gross cardiac slowing. In group *B*, $CO_2$ pressure tended to show a gradual fall concomitant with the hyperpnoea, followed late in the experiment by a slight rise. No high values were recorded in this group, and as has been noted already respiratory failure did not occur. Three

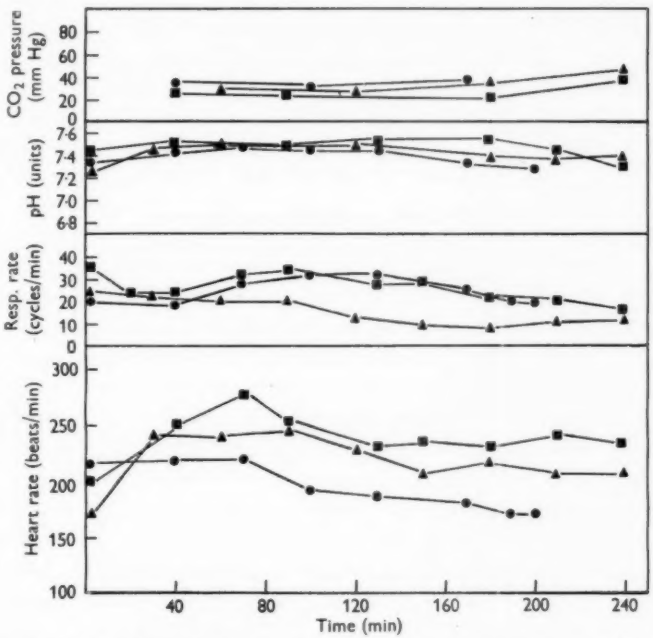


Fig. 2. Graphs (from above down) of calculated $CO_2$ pressure and pH of mixed venous blood from right atrium, respiratory rate and heart rate plotted against time from beginning of compression, for three consecutive experiments on cats anaesthetized with Nembutal and exposed to $O_2$ under a pressure of 6 atm. ■, cat 15; ▲, cat 17; ●, cat 19.

animals showed some degree of cardiac slowing without any rise in $CO_2$ pressure. These changes in cardiac and respiratory rates, and pH and $CO_2$ pressure of mixed venous blood are illustrated graphically in Figs. 1 and 2 for 3 consecutive experiments from each group.

*Oxygen content of blood.* When, neglecting time of sampling, all blood samples were taken together and compared, it was found that there was no clear difference between the two groups in $O_2$ content of arterial blood samples, but that the $O_2$ content of the mixed venous blood samples from group *B* was lower than that from group *A* by 3 vol. % on average.

*Effects of cutting both sinus nerves.* In 4 experiments, 2 with each

anaesthetic, both sinus nerves were cut and after being tested by carotid compression and observing the blood pressure, the animals were exposed to oxygen in the usual way. This procedure did not apparently modify the results which have been described above. Both animals given chloralose

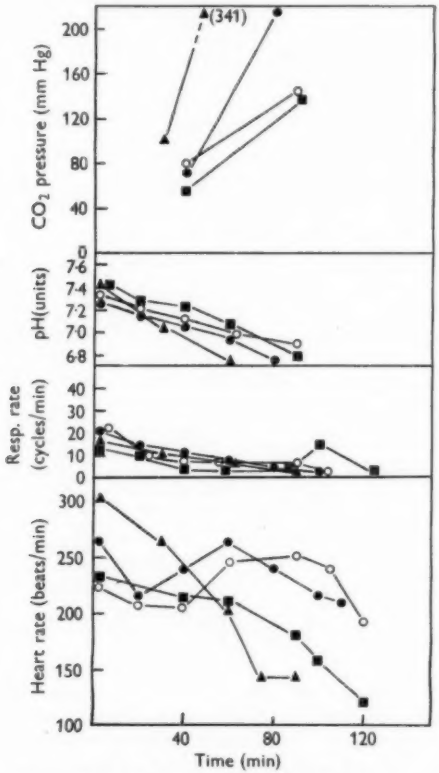


Fig. 3. Graphs (from above down) of calculated $CO_2$ pressure and pH of mixed venous blood from right atrium, respiratory rate and heart rate plotted against time from beginning of compression for four bilaterally vagotomized cats exposed to $O_2$ under a pressure of 6 atm. Cat 32, ■, and cat 33, ▲, were anaesthetized with chloralose and urethane; cat 34, ●, and cat 35, ○, were anaesthetized with Nembutal.

and urethane suffered cardiac and respiratory failure in less than 2 hr, one of them exhibiting convulsive movements. Neither Nembutal-treated animal became convulsed, nor showed any evidence of respiratory failure within 3 hr, but in one of them the heart had slowed by about one third at the end of that time. After section of the sinus nerves heparinization of the animal was undesirable, and thus blood samples were not taken

in these 4 experiments and in the 6 experiments described immediately below.

*Effects of cutting both sinus nerves and both vagus nerves.* In 6 experiments, 3 with each anaesthetic, both sinus nerves and both vagus nerves were cut. All three cats given chloralose and urethane showed convulsive movements, and heart and respiration failed within 80–100 min. A fall in heart rate was, however, not obvious initially but took place very suddenly after 80–90 min. No Nembutal-treated cat became convulsed, two showed no apparent deterioration after 3½ hr and in one there was a slowing of heart and respiratory rates of about one third after 150 min.

*Effects of cutting both vagus nerves only.* In 11 experiments, 4 with chloralose and urethane and 7 with Nembutal, both vagus nerves were cut. In all 4 chloralose experiments, and in 3 with barbiturate, blood samples were taken for analysis. All four chloralose-treated animals had convulsive movements, and three of the four showed evidence of circulatory and respiratory collapse in 1–2 hr, with a rapid and severe fall in blood pH, and a rise in $CO_2$ pressure to very high levels, of the order of 100–200 mm Hg, relatively early after exposure to $O_2$ had begun. Of the seven cats given Nembutal five showed evidence of respiratory failure; the heart rates tended to slow slightly, then to increase, and then to slow more abruptly as respiration failed, the pattern thus differing from those in chloralose-treated animals. As respiration failed, blood pH decreased rapidly and $CO_2$ pressure rose steeply. These were the only Nembutal-treated cats of the whole series which behaved in this way, and three exhibited convulsive jerking movements. These results are illustrated for two animals from each group in Fig. 3.

## DISCUSSION

The results which have just been described confirm and extend those obtained when using rats and presented in an earlier paper (Taylor, 1958). There was the same difference in the cat as in the rat between animals anaesthetized with chloralose and urethane and those given barbiturate, in the time taken for an appreciable degree of cardiac and respiratory slowing to develop. The role of the vagus nerves in the early onset of bradycardia, emphasized by Whitehorn & Bean (1952) was again difficult to determine on account of early respiratory failure in the majority of vagotomized animals. In the three cats anaesthetized with chloralose and urethane and subjected to section of both sinus nerves and bilateral vagotomy early cardiac slowing was less obvious than usual. Nevertheless, some degree of cardiac slowing in the absence of any large change in respiratory rate certainly took place in three vagotomized animals in 60–80 min from the beginning of exposure to $O_2$. Conversely, it is hard to

believe that the absence of cardiac slowing in animals given Nembutal, but otherwise intact, is due to abolition of vagal function by the anaesthetic, when section of both vagi in such animals resulted in clear and immediate changes in respiration. As in the earlier experiments on rats (Taylor, 1958) there was no apparent connexion between changes in heart rate and changes in arterial blood pressure and section of the sinus nerves with or without vagal section did not alter the differences between chloralose- and Nembutal-treated animals.

The results of the blood gas analyses seem to show clearly that considerable cardiac slowing and some degree at least of respiratory slowing can precede any changes in $CO_2$ pressure as indicated by the measurements made. Bean (1945) has stated that $CO_2$ pressure of arterial blood is not necessarily an indication of that obtaining in the tissues. The values obtained in the present experiments from analyses of arterial and mixed venous blood agree well with those for arterial and jugular-vein blood of Lambertsen and his colleagues, who have discussed in great detail the relation between arterial and venous blood $CO_2$ pressures and 'central' $CO_2$ pressure (Lambersten, Kough, Cooper, Emmel, Loeschke & Schmidt, 1953*a*, *b*; Lambertsen, Stroud, Ewing & Mack, 1953). When respiration continues to slow, however, a point seems to be reached when $CO_2$ clearance is no longer adequate and the $CO_2$ pressure then rises, as occurred eventually in the majority of animals given chloralose and urethane. The convulsions so frequently observed in these animals are equally unlikely to be due to a primary rise in $CO_2$ pressure, since they regularly occurred before any significant change in the $CO_2$ pressure was observed, although they presumably contributed to the $CO_2$ load in such cases.

In Nembutal-treated animals, on the other hand, the early changes in pH and total $CO_2$, and thus in $CO_2$ pressure, pointed to a respiratory alkalosis as a result of the hyperpnoea, and only in those cases where vagotomy resulted in an initial and independent impediment to respiration was there a failure of adequate $CO_2$ excretion.

It was not possible to obtain blood from the jugular vein by the methods used in these experiments and, failing such, it is impossible to say whether or not the observed difference in $O_2$ content of the mixed venous blood between the groups implied a difference in cerebral capillary $O_2$ pressure, the importance of which has been stressed by Lambertsen *et al.* (1953*a*). The greater desaturation of the mixed venous blood after Nembutal may have been a result of effort involved in the hyperpnoea, although it is not obvious that this activity entailed a greater $O_2$ consumption than the convulsions of chloralose.

It is beyond the purpose of this paper to discuss why animals given Nembutal should exhibit early respiratory failure after bilateral vago-

tomy but not when this procedure was combined with bilateral section of the sinus nerves, especially as no control observations were made in animals not exposed to oxygen. Von Euler & Söderberg (1952) stated that chloralose strongly depressed the central effects of $CO_2$ and the results, as a whole, seem to show that the key to the differences between the two groups probably lies, as Moruzzi (1955) implied, in a difference in the central control of respiration under the two anaesthetics. Further investigation might therefore be most usefully devoted to studying the influence of a combination of anaesthetic and $O_2$ under high pressure on the functioning of cerebral cortex and respiratory centre.

SUMMARY

1. The effects of oxygen under a pressure of 6 atmospheres on cardiac and respiratory rates, and on the $CO_2$ pressure and pH of mixed venous and arterial blood samples, were studied in cats anaesthetized with chloralose and urethane and with Nembutal.

2. There was an obvious difference, confirming earlier experiments on rats, in the average time required for an appreciable degree of slowing of the heart and respiration to develop, the changes occurring much sooner in cats given Nembutal.

3. These differences were not affected by bilateral section of the sinus nerves with or without bilateral vagotomy.

4. There was no evidence of a rise in $CO_2$ pressure in the blood before the onset of respiratory failure.

5. When bilateral vagotomy only was performed the differences between the two groups largely disappeared as a result of early respiratory failure in the cats given Nembutal. In both groups of animals $CO_2$ pressure in the mixed venous blood rose to very high levels.

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# FACTORS AFFECTING MILK RELEASE IN THE DOG AND THE QUANTITY OF OXYTOCIN LIBERATED BY SUCKLING

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In the many species studied oxytocin and vasopressin of the pars nervosa are liberated by a wide variety of stimuli (Harris, 1955), and in all instances there is evidence that the liberation of oxytocin is accompanied by the simultaneous release of vasopressin (ADH). Further, more oxytocin than ADH is released as the result of any one stimulus (Abrahams & Pickford, 1954; Harris, 1955). The recorded ratios of ADH:oxytocin vary from 1:4 to 1:100 in different species (Harris, 1955), but so far no reports have appeared showing whether this ratio varies with the stimulus applied or is constant in a species or individual. Previous work on conscious bitches (Abrahams & Pickford, 1954) showed that the injection of a small volume of hypertonic NaCl solution into the exteriorized carotid artery during water diuresis caused an increase in uterine activity and an antidiuresis which could be matched by the intravenous injection of approximately 3 m-u. Pitressin (vasopressin: Parke, Davis) and 80–100 m-u. Pitocin (oxytocin; Parke, Davis); that is, the ADH:oxytocin ratio lay between 1:25 and 1:30. It was decided to attempt an assay of the amount of oxytocin liberated in the bitch by suckling to determine what was the ratio of ADH:oxytocin on the application of this physiological stimulus and, as opportunity offered, to make some other observations on milk release. A short summary of some of the results has already been published (Pickford, 1959).

## METHODS

Observations were made on three bitches; Lottie (15 kg), Misty (13 kg) and Bianca (24 kg), during the period from 6 days to 5 weeks after whelping. During observations the animal was lying on its side. All three animals were well used to the routine. Two dogs, Lottie and Bianca, were each provided with a permanently exteriorized carotid artery whose sinus had been denervated. The routine was as follows: the animal was hydrated at 12 noon with 250 ml. water given by stomach tube, and at this time the puppies were separated from the mother; water diuresis was induced at 2·15 p.m. by the administration of 250–350 ml. water according to the size of the dog. Urine was collected into graduated tubes by means of a rubber catheter inserted into the bladder through the urethra. The dead space was reduced to a minimum. Urine was collected over 10 min periods, the volume noted, and the samples later analysed for their Na, K and Cl content, except when hypertonic NaCl solutions had been administered. No experimental manoeuvres such as injections

were performed, nor were the pups allowed access, until at least 35 min after administration of water. Most of the observations were made when the animals were conscious, but in some instances anaesthesia was induced with sodium thiopentone administered intravenously at a time when water diuresis was established. Usually the pups were allowed to feed one at a time, and only occasionally all together. They were weighed rapidly to the nearest 0·5 g before and after each feed or attempted feed, and their hind quarters were wrapped in a duster or inserted into a small bag so that faeces or urine passed during feeding should not be lost and falsify the weighings. It was possible to tell by the behaviour of the pups whether they were obtaining milk; if successful they soon became quiet, but if unsuccessful they moved frenziedly from one teat to another in quick succession.

In Bianca, a 24 kg Labrador, it was possible to record the milk ejection response to various stimuli at a time when she was unanaesthetized. The dog seemed undisturbed by the insertion of a fine polythene cannula into a milk duct. The cannula was connected to a 5 ml. volume recorder on which was a light pointer writing on smoked paper. The cannula was filled with 5% potassium citrate solution and was held in place by gently pinching the teat between finger and thumb. On any one day, with few exceptions, observations were made from the same teat.

One dog, Lottie, is still alive. After the other two had been killed, routine histological examination was made in each of the hypothalamus and pituitary gland and of both kidneys and uterine horns. No abnormalities were noted.

Urinary Na and K were estimated by flame photometry, and Cl by the method of Prout Winter as described by Cole (1919).

The drugs used were Pitressin, Pitocin and adrenalin and Syntocinon (oxytocin; Sandoz Ltd). No difference was noted in the actions of Pitocin and Syntocinon and they will be referred to indiscriminately as oxytocin.

## RESULTS

### *Effect of suckling on urine flow and composition, and assay of hormones liberated*

Of the three dogs used, Lottie always voluntarily fed her pups when under observation, Misty did so when all six were alllowed to feed, and Bianca always refused. It was found that 5–6 min was sufficient time for a puppy to empty one gland and that, with Lottie and Misty, the weight gained varied between 20 and 25 g as the result of one such feed. In Lottie's case the number of pups allowed to feed made no difference to the result. Fig. 1*A* shows a typical response of Lottie to suckling, a small antidiuresis quickly set in after suckling began (in this instance suckling five puppies), and was accompanied by a rise in the excretion of Na and Cl. For comparison a typical control water diuresis may be seen in Fig. 3*B*. In Misty the mammary response depended on the number of pups allowed to feed at one time. On no occasion out of fifteen was a lone pup voluntarily given milk, but all three times when six pups were suckled they all made an almost maximal weight gain. As in the case of Lottie, milk release was accompanied by a small antidiuresis (Fig. 2) and a slight rise in Na and Cl excretion. A rise in electrolyte excretion was never seen during the course of an undisturbed water diuresis (Ali, Cross & Pickford, 1958).

The degree of antidiuresis caused by suckling in Lottie varied slightly on different days and could be matched by the intravenous injection of 0·1–0·5 m-u. Pitressin (Fig. 1*B*). In Misty with six pups being suckled the antidiuresis was also similar to that following the intravenous injection of up to 0·1–0·5 m-u. Pitressin (Fig. 2). On the other hand, up to 50 m-u. oxytocin never caused antidiuresis in any of the dogs.

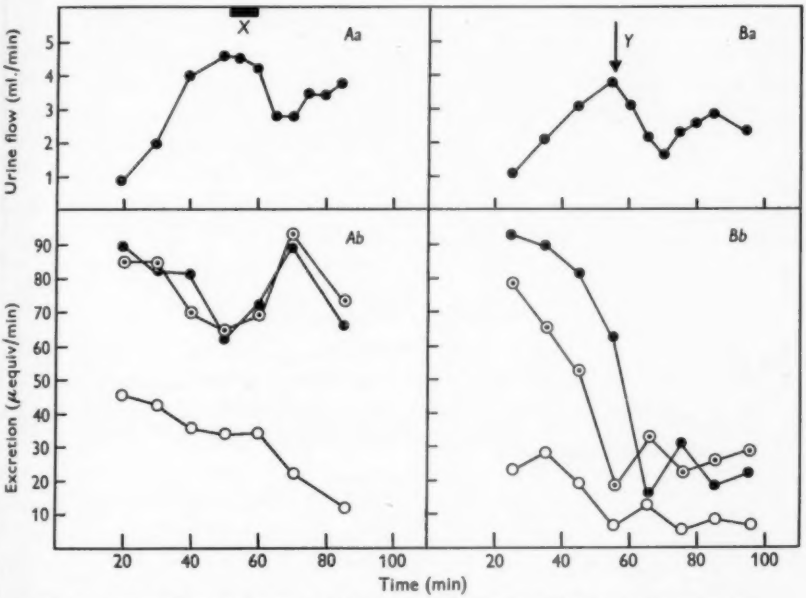


Fig. 1. Effect of suckling and ADH on water diuresis and electrolyte excretion in a dog (Lottie). In both *A* and *B*, 300 ml. water by mouth at zero time. *Aa* and *Ba*, rates of urine flow (ml./min); *Ab* and *Bb*, rate of excretion of Na (●), K (○) and Cl (⊙) (μequiv/min). In *A*, at *X*, five puppies were allowed to suck for 7 min. In *B*, at *Y*, 0·5 m-u. ADH injected intravenously.

For assaying the amount of oxytocin necessary for a full let-down of milk, Lottie and Misty were anaesthetized with intravenous sodium thiopentone. This drug was chosen because of the rapid onset and disappearance of its action and because it seemed not to interfere with water diuresis (Fig. 3*A*). It is suggested that the absence of an inhibition of urine flow was in part related to the fact that the dogs were well used to receiving intravenous injections, and in part, that the rapidity of action of sodium thiopentone allowed no time for emotional disturbance. In support of the latter suggestion it was noticed that when the rate of urine flow was still high during recovery from anaesthesia, some inhibition occurred at

that time. The weight gained by the pups was used as a criterion of the adequacy of milk ejection. As soon as the mother was anaesthetized a pup was allowed to feed and, as expected, was unable to obtain milk; nor did its efforts affect the excretion of urine. After weighing, it was returned to the mother immediately following the injection of oxytocin and weighed

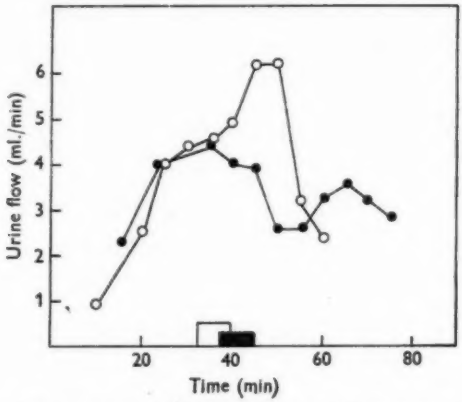


Fig. 2. Effect of suckling one or six puppies on water diuresis in a dog (Misty). 250 ml. water given by mouth at zero time. In curve ○—○ one puppy was allowed to suck for 7 min (open block on abscissa). In curve ●—● six puppies allowed to suck (filled block).

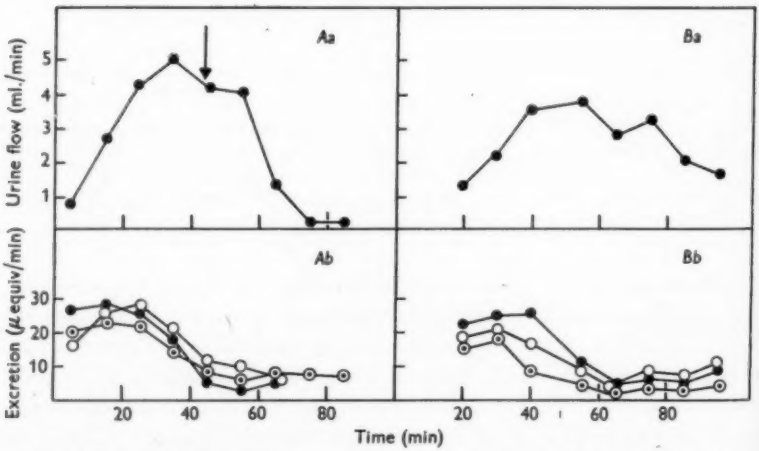


Fig. 3. Effect of sodium thiopentone on water diuresis and electrolyte excretion in a dog (Lottie). In both *A* and *B* 300 ml. water given by mouth at zero time. *Aa* and *Ba*, rates of urine flow (ml./min). *Ab* and *Bb*, rate of excretion of Na (●), K (○) and Cl (⊙) (μequiv/min). In *A* at arrow, anaesthesia rapidly induced with intravenous sodium thiopentone. *B*, undisturbed water diuresis.

again 6–7 min later. In Lottie on four occasions it was found that 5 m-u. oxytocin was insufficient to allow pups to gain any weight; in one test out of three 10 m-u. allowed about half the possible weight gain; on four out of four occasions 15 m-u. oxytocin permitted the full weight gain. Some of these observations were made consecutively. Thus, on one day 10 m-u. oxytocin given immediately anaesthesia was complete was wholly ineffective, 5 m-u. given 12 min later was also ineffective, but 15 m-u. given after a further 13 min permitted a full weight gain by a puppy. On five occasions Misty needed 15 m-u. oxytocin for full milk release, both when she was anaesthetized and on those days when she was conscious and refused to give milk to a single pup. Twice, under anaesthesia, 10 m-u. oxytocin was enough for a full weight gain.

In none of the dogs did 0·5 m-u. Pitressin, or even 2 m-u., allow pups to gain any weight. The above results indicate that, in the circumstances, the ratio of ADH:oxytocin released was approximately 1:30, and that the quantity of ADH released was not enough to cause a let-down of milk.

*Milk ejection response to administration of oxytocin*

The observations in this section were made on Bianca. Since this was the animal which never gave milk voluntarily when under observation, anaesthesia was unnecessary, and oxytocin had to be injected in order to find the maximal possible weight gain of a pup allowed to suck one breast for at least 6 min. The weight gain was found to be 25–30 g and the dose of oxytocin needed during the first 3 weeks of lactation varied from 15 to 20 m-u. The administration of more than 20 m-u. did not enable a pup to gain more weight, and after less oxytocin the weight gain was submaximal. Figure 4 shows the milk ejection response to 20 and 10 m-u. oxytocin from one milk duct, at times when a puppy was allowed to suck at first one and then another teat. After 20 m-u. oxytocin the pup gained 30 g weight, and after 10 m-u. another pup gained 8 g. This result correlated with records of milk ejection; for instance, in Fig. 5 it can be seen that the ejection responses bore a close arithmetical relationship to the dose of oxytocin given when this ranged from 10 to 20 m-u. These observations are typical of several made during the 2nd and 3rd weeks of lactation, and the direct dose–response relationship held from 5 to 20 m-u. oxytocin.

It was noticed during the 4th and 5th weeks of lactation that milk ejection could be induced by smaller doses of oxytocin than formerly and that no clear dose–response relationship was apparent; for instance, 2·5 m-u. oxytocin caused a greater milk ejection than had 20 m-u. previously, and 5 m-u. permitted a full weight gain. It is not known whether this was a genuine increase in sensitivity or was due to conditioning. Another fact noticed in the later weeks was that the insertion of the cannula was

sometimes a stimulus sufficient to cause milk ejection. This had not occurred in the first 3 weeks and suggests that there was indeed some increase in reactivity of the myo-epithelial cells. At no time did tapping or handling the gland cause milk ejection (Cross, 1954) in any of the dogs.

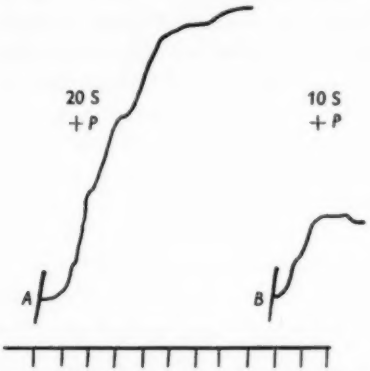


Fig. 4. Tracing of record of milk ejection from a single milk duct in a conscious dog (Bianca). At least 15 min between injections. At *A*, 20 m-u. oxytocin (S) intravenously and a puppy (*P*) put to another teat. At *B*, 10 m-u. oxytocin and a puppy put to a full gland. In first instance pup gained 30 g weight, in second instance 8 g. Irregularities in curves due to activity of puppies. Time marker, 30 sec.

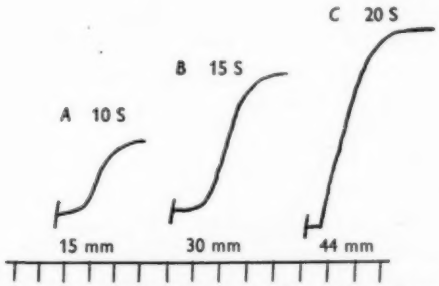


Fig. 5. Tracing of record of milk ejection from a single milk duct in a conscious dog (Bianca). At *A*, *B* and *C*, 10, 15 and 20 m-u. oxytocin (S) respectively given intravenously. Values below curves show height in mm of the original records. At least 15 min between injections. Time marker, 30 sec.

*Effect of hypertonic NaCl solution on milk ejection and urine volume*

The effect of applying an osmotic stimulus, namely, the injection of 1·7 M-NaCl solution into the carotid artery, was tested mainly on Lottie. Control injections of NaCl solution 0·9 % (w/v) were without effect on milk ejection or urine flow in this dog or in Bianca (Figs. 7*A* and *B*). In Lottie under sodium thiopentone anaesthesia 5 and 3 ml. of the hypertonic

NaCl solution caused pups being suckled to make a maximal weight gain and resulted in prolonged antidiuresis. On two separate days 1 ml. of the NaCl solution caused a considerable antidiuresis (Fig. 6) and the pups gained 20 and 12 g weight. It is not clear why the weight gain differed so widely on the two occasions. There was insufficient time during lactation to test the response to 0·5 ml. hypertonic NaCl solution, but 5 weeks after

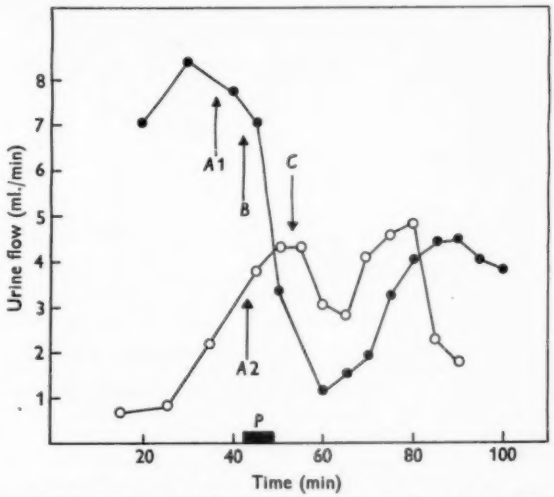


Fig. 6. Effect of hypertonic NaCl solution on water diuresis in a dog (Lottie). 300 ml. water by mouth at zero time. At *A* 1 and *A* 2 anaesthesia rapidly induced with intravenous sodium thiopentone. In curve ●—●, at *B*, 1 ml. 1·7M-NaCl solution injected into carotid artery; a puppy allowed to feed during time marked *P* gained 20 g weight. In curve ○—○, at *C*, 0·5 ml. 1·7M-NaCl solution into the carotid artery.

the end of lactation this volume induced an antidiuresis only a little less than that seen following 0·5 m-u. ADH (Fig. 6). In Bianca when conscious the intracarotid injection of 4 and 5 ml. of 1·7M-NCl solution had no effect on either milk ejection or rate of urine flow. In Misty, also when conscious, when 11 ml. of 1·7M-NaCl solution was injected intravenously a puppy made a weight gain of 10 g, but up to 5 ml. given intravenously was ineffective in all the dogs. No other observations were made on the effect of intravenous injections of hypertonic solutions.

### *Effect of acetylcholine on milk ejection*

These observations were made on Bianca. The effect of acetylcholine (ACh) alone was tested on three separate days, and it was found that 100 μg in 1 ml. NaCl solution 0·9 % (w/v) injected into the carotid artery

caused a small but definite milk-ejection response. A large milk ejection followed 200 $\mu$g ACh (Fig. 7*D*) and a full weight gain was made by a feeding pup. The milk ejection response was similar to that following the intravenous injection of 20 m-u. oxytocin (Fig. 7*F*). Given intravenously, 200 $\mu$g ACh caused no milk ejection (Fig. 7*E*). On no occasion was antidiuresis seen.

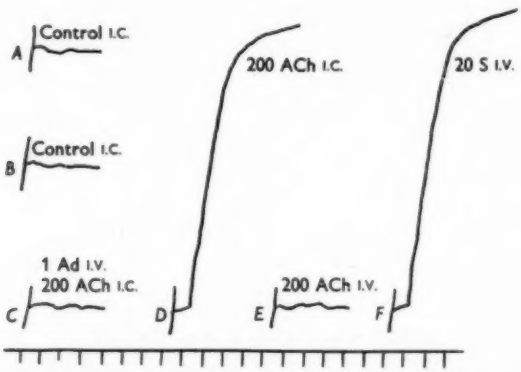


Fig. 7. Tracing of milk ejection record from a single milk duct in conscious dog (Bianca); effect of ACh and adrenaline. At least 15 min between injections. At *A* and *B*, 1 ml. 0·9 % NaCl solution into the carotid artery. At *C*, 1 $\mu$g adrenaline intravenously and 10 sec later 200 $\mu$g ACh into carotid artery. At *D*, 200 $\mu$g ACh into carotid artery. At *E*, 200 $\mu$g ACh intravenously. At *F*, 20 m-u. oxytocin intravenously. Ad, adrenaline; I.V., intravenous; I.C., intracarotid; S, oxytocin. Time marker, 30 sec.

### *Effect of adrenaline on milk ejection*

These observations, too, were made on Bianca, and were of two kinds. The effect of a preliminary injection of adrenaline on the response to oxytocin was first tested, and then its effect on the response to an intracarotid injection of ACh. In all instances adrenaline was injected intravenously in 0·5 ml. NaCl solution 0·9 % (w/v) 10 sec before the oxytocin or ACh. Figure 8*B* shows that when 0·5 $\mu$g adrenaline was given before 25 m-u. oxytocin (i.e. a supramaximal dose) there was a just perceptible milk-ejection response (Fig. 8*E*), and there was a partial response on two occasions when 0·25 $\mu$g adrenaline was injected (Fig. 8*C* and *D*). Figure 8*F* shows that at the end of the afternoon 25 m-u. oxytocin was still able to cause a full milk ejection from the duct under observation. The inhibitory power of adrenaline was also exerted on the response to ACh. In Fig. 7*C* it is shown that no milk ejection followed when 1 $\mu$g adrenaline was given 10 sec before the intracarotid injection of 200 $\mu$g ACh. Some 15 min later 200 $\mu$g ACh caused a normal milk ejection (Fig. 7*D*).

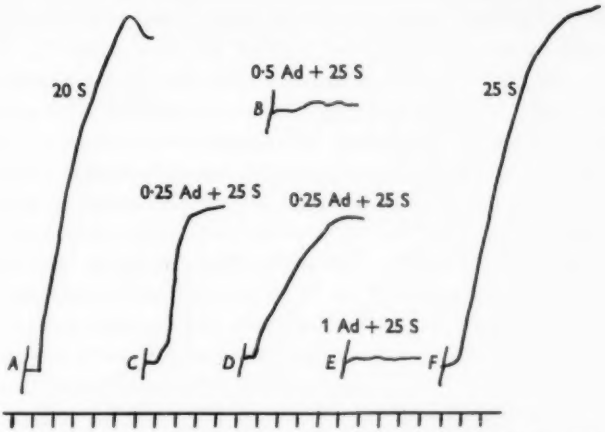


Fig. 8. Tracing of milk ejection record from a single milk duct in conscious dog (Bianca); effect of adrenaline (Ad) on response to oxytocin (S). At least 15 min between injections, all given intravenously. At *A*, 20 m-u. oxytocin. At *B*, 0·5 $\mu$g adrenaline and 10 sec later 25 m-u. oxytocin. At *C*, and *D*, 0·25 $\mu$g adrenaline and 10 sec later 25 m-u. oxytocin. At *E*, 1 $\mu$g adrenaline and 10 sec later 25 m-u. oxytocin. At *F*, 25 m-u. oxytocin. Time marker, 30 sec.

## DISCUSSION

The results described show that puppies made a maximal weight gain on being suckled after the injection of 15 m-u. oxytocin into dogs of 13–15 kg body weight; and with a 24 kg dog, after 20 m-u. oxytocin. Only in the two smaller dogs was it possible to make observations on the antidiuresis accompanying suckling, and it was found to be of the same order as that following injection of not more than 0·5 m-u. Pitressin. During the antidiuresis there was some rise in rates of excretion of Na and Cl. The findings on antidiuresis are the same as those of Kalliala, Karvonen & Leppänen (1952). Thus the ADH:oxytocin ratio was approximately 1:30, that is, of the same order as that found when applying an osmotic stimulus and using the uterus as the assay organ. One dog gave milk when suckling six pups but not when suckling one. It is impossible to say whether this was due to the greater stimulus in the first instance, or to an emotional inhibition in the second.

The experiments with ACh confirmed those made earlier with the uterus as the test organ (Abrahams & Pickford, 1954), namely, that ACh causes the release of oxytocin, as of vasopressin, by action at a site in the central nervous system. Further, the dog was found to be like both the goat (Andersson, 1951) and the rabbit (Holland, Cross & Sawyer, 1959) in that an osmotic stimulus induced the let-down of milk. Thus observations

on both the mammary glands and the uterus lead to the same conclusion (Abrahams & Pickford, 1954), that ACh and osmotic stimuli release both oxytocin and vasopressin.

Cross (1953) found that in the rabbit adrenaline, by a peripheral action, prevented the response to a rapidly following injection of oxytocin. In the dog, too, adrenaline prevented the response to oxytocin, whether the oxytocin was given by intravenous injection or released endogenously by administration of ACh. However, even without making allowance for differences in body weight, the dog appears to be more sensitive to oxytocin than the rabbit, and if body weight allowance is made, the dog is also more sensitive to adrenaline. Thus, in dogs 15–20 m-u. oxytocin caused a maximal milk release during the first half of lactation, and in the later part as little as 5 m-u. oxytocin was sufficient. The response to oxytocin was almost wholly prevented by 0·5 $\mu$g adrenaline and entirely suppressed by 1 $\mu$g. The quantities found by Cross & van Dyke (1953) and Cross (1954) for the rabbit were 50 m-u. oxytocin and 1 $\mu$g adrenaline. It is not possible to compare the sensitivity of dogs and rabbits to hypertonic NaCl solution, since Holland *et al.* (1959) used a 1·5 M solution, and in the present experiments a 1·7 M solution was used.

A question arises from the work on milk ejection in Bianca. On injection into the carotid artery of 200 $\mu$g ACh the quantity of oxytocin released was assayed as 20 m-u.; further, 5 ml. 1·7 M-NaCl solution given into the carotid artery caused no observable liberation of either of the pars nervosa hormones. These results contrast with earlier studies in which the uterus and kidney were used as test organs (Abrahams & Pickford, 1954), when it was found that both 200 $\mu$g ACh and 2 ml. or less of 1·7 M-NaCl solution caused the release of approximately 3 m-u. ADH and 80–100 m-u. oxytocin. In the present work, too, Lottie responded by antidiuresis to as little as 0·5 ml. 1·7 M-NaCl solution. The difference between the past results and those on Bianca could, perhaps, be connected with the fact that this dog had a large and muscular head, in other words, a vascular bed of considerable volume which would lower the concentration of ACh or NaCl reaching the central active site. It seemed unlikely that the present results were related in some way to the lactational state, for instance, the greater than normal fluid turnover; or to the different organ used for the purpose of assay. Some observations made on Bianca before she was pregnant showed that 1 m-u. Pitressin gave rise to a barely perceptible antidiuresis, that 3 m-u. caused a brief 50 % reduction in the rate of urine flow, and 4 m-u. Pitressin caused a 66 % reduction. Two-and-one-half months after the end of lactation these experiments were repeated and the results confirmed, and other observations were made. It was found that in order to cause antidiuresis of the same order as that due to 4 m-u.

Pitressin it was necessary to inject at least 8 ml. of 1·7 M-NaCl solution into the carotid artery, and to inject it in not more than 0·75 min. Further, it was confirmed that 200 $\mu$g ACh given into the carotid artery caused no antidiuresis, and that 300 and 400 $\mu$g were also ineffective. A transitory 50% inhibition of the rate of urine flow followed the injection of 600 $\mu$g ACh. All observations in which ACh was used were preceded by a subcutaneous injection of 2 mg atropine sulphate. From these results it seemed that at all times this dog was relatively insensitive to the intracarotid injection of ACh, and moderately insensitive to that of hypertonic NaCl solution. Further confirmation was sought in the following way. Five months after whelping, the dog was ovariectomized and a left uterine fistula made (Abrahams & Pickford, 1954). The uterus developed spontaneous activity 10 days after operation and observations were begun 4 days later. Uterine activity sometimes, but not always, increased slightly on the intravenous injection of 50 m-u. oxytocin, but never following 20 m-u. oxytocin. The rapid intracarotid injection of 8 ml. 1·7 M-NaCl solution caused, on two separate days, a small but definite increase in size and frequency of uterine contractions. Following subcutaneous atropine there was no uterine response to the intracarotid injection of 200 $\mu$g ACh; on one occasion 300 $\mu$g ACh caused an increase in activity less than that due to 50 m-u. oxytocin, and 500 $\mu$g ACh induced a moderate increase in contraction frequency for 7 min. With a dose of ACh as large as 500 $\mu$g some direct peripheral action cannot be discounted. Thus, all results are similar in showing that, in order to cause a release of ADH and oxytocin, this dog needed relatively large amounts of ACh or NaCl to be injected into the carotid artery. Probably then, the release of only 20 m-u. oxytocin after an injection of 200 $\mu$g ACh was normal for this dog, and was not related to the lactational state nor to the use of the mammary glands rather than the uterus for assay purposes. Since it is probable that rather less than 1 m-u. ADH would have been released with 20 m-u. oxytocin, it is not surprising that no antidiuresis was seen. The results obtained from this dog, therefore, invalidate neither the earlier conclusions nor the present ones, that after both an osmotic stimulus and ACh, as well as on suckling, the pars nervosa of the dog releases oxytocin and ADH simultaneously, and that they are released in a ratio of one part of ADH to about 30 parts of oxytocin. The only result which seemed to give a different ratio was that obtained from Lottie under anaesthesia, when following an intracarotid injection of 1 ml. 1·7 M-NaCl solution the antidiuresis was far greater than could be due to 0·5 m-u. ADH, yet a puppy made only a small weight gain on being suckled. This isolated result may be of significance, but it seems more likely that some inhibition prevented the full mammary response; there may have been a central

stimulation of sympathetic areas and a release of adrenaline, as was seen in the rabbit by Holland *et al.* (1959).


## SUMMARY

1. Simultaneous observations on milk release and urine flow were made on three bitches during lactation.

2. Puppies made a maximal weight gain on being suckled after the intravenous injection of 15–20 m-u. oxytocin. Voluntary milk release was accompanied by an antidiuresis which could be matched by the intravenous injection of 0·1–0·5 m-u. vasopressin.

3. During the first 3 weeks of lactation there was, in the conscious dog, an arithmetical relation between the milk ejection response and dose of oxytocin up to 20 m-u. oxytocin.

4. The intracarotid injection of either a solution of acetylcholine or of hypertonic NaCl caused milk ejection.

5. The intravenous injection of 1 $\mu$g adrenaline immediately before an injection of oxytocin or acetylcholine wholly prevented the milk ejection response to either of these substances.


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# THE ACUTE EFFECTS OF INJECTION OF THYROTROPHIC HORMONE OR OF ELECTRICAL STIMULATION OF THE HYPOTHALAMUS ON THYROID ACTIVITY

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Harris & Woods (1958) reported that electrical stimulation of the anterior hypothalamus results in increased activity of the thyroid gland. The remote control method of stimulation was usually applied for 48-hr periods to rabbits in which changes of thyroid function were assessed by studies of the rate of release of $^{131}I$ from the thyroid. Each experiment took about 10 days to perform. For further studies it was desirable to devise a method of observing acute changes in thyroid activity in experiments lasting a few hours. The present report describes such a method and gives the results obtained following (*a*) injections of thyrotrophic preparations (TSH) or (*b*) electrical stimulation of various hypothalamic regions.

The ideal method of measuring the activity of endocrine glands appears to be to measure the rate of secretion of hormone into the blood stream; that is, to measure the hormone content of the arterial blood to, and the venous blood from, the gland and the rate of blood flow through it. Various attempts which approximate to the ideal method, in that measurements were made of the hormone content of the glandular venous blood, have been reported (adrenal medulla, Satake, Sugawara & Watanabe, 1927; Brucke, Kaindl & Mayer, 1952; Dunér, 1953; Folkow & von Euler, 1954; Satake, 1955; adrenal cortex, Vogt, 1943, 1944; Bush, 1951; Nelson & Hume, 1955; Reif & Longwell, 1958; neurohypophysis, Andersson, 1951; Ginsburgh & Heller, 1953; Fitzpatrick & Hughes, 1957; testis, Samuels, 1957). Söderberg (1958) cannulated thyroid veins in anaesthetized cats and rabbits and presented clear results on the rate of thyroid blood flow, rate of iodine uptake and rate of secretion of labelled thyroid hormone. The techniques developed by Söderberg were applicable to observations extending over periods of seconds to minutes and were used to determine the effects on thyroid blood flow and activity of interference with the nerve supply to the gland or the administration of various substances.

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Other experiments involving collection of venous blood from the thyroid gland have been made in dogs (Ackerman & Arons, 1958) and rabbits (Monkus & Reineke, 1958).

In the present study on rabbits a technique has been developed which allows collection of small blood samples to be made at quarter-hour intervals from a cannulated thyroid vein and a saphenous artery. This procedure has been used to investigate the effects of administration of thyrotrophic hormone and of electrical stimulation of the hypothalamus on the release of radioactive hormone from the thyroid gland over periods of 8–10 hr.

## METHODS

Adult female chinchilla rabbits, 2·0–4·0 kg body weight, were used. For a few days before the experiment the animals were maintained at a constant room temperature of 27–29° C, under constant conditions of lighting, and fed on a standard diet of pellets (M.R.C. diet 18, supplied by A. C. Taylor Ltd, London) and tap water, *ad lib.* Two to four days before the experiment each animal received 20–80 $\mu$c $^{131}$I by subcutaneous injection.

### *Operative procedures*

In the early experiments in which the effect of TSH injections was studied, pentobarbitone sodium (Nembutal, Abbott Laboratories) 0·6 ml./kg body wt. I.V. was used as the anaesthetic. In the later experiments, which included those dealing with electrical stimulation of the hypothalamus, the animals were anaesthetized by intravenous injection of a mixture of 2 % chloralose and 10 % urethane (2·5–3·5 ml./kg body wt.). In all cases the trachea was intubated and the anaesthesia supplemented as required with ether. After anaesthetizing the radioactivity in the thyroid region was measured in the manner described by Brown-Grant, von Euler, Harris & Reichlin (1954). The rabbit was then fixed in a head holder (described and illustrated by Fortier, Harris & McDonald, 1957) and placed in a supine position, and a thermometer was inserted in the vagina. A mid-line incision from the cricoid to the upper end of the manubrium sterni exposed the infra-hyoid muscles. By gently retracting these muscles laterally the two inferior thyroid veins, which run on the postero-lateral border of the trachea, were exposed. In the majority of animals the left vein was found to be larger than the right. In all manipulations care was taken to avoid disturbance in the region of the thyroid gland, which was left covered by muscle and fascia throughout the experiment. After ligating and cutting the sternomastoid muscle and the infrahyoid muscles on the side of the larger vein, a fine suture inserted in the pretracheal fascia served to retract the vein clearly into view. Further dissection was performed with the aid of a Zeiss operation microscope giving a magnification of about × 6. The vein was gently freed from surrounding fascia and fat, and two fine cotton ligatures placed around a region free of tributaries and as far removed from the thyroid gland as possible. (For a description of the thyroid blood vessels in the rabbit, see under Results.) The saphenous arteries in the two lower limbs were exposed and ligatures placed in position around these vessels. After all bleeding had been carefully controlled, heparin 6000 i.u. (Liquimin; Roche Products Ltd) was given intravenously, followed by 1000 i.u. at hourly intervals. Fine polythene cannulae were then tied into each saphenous artery, one being connected to a mercury manometer and the other clamped and used for obtaining arterial blood samples. A third polythene cannula, with a tapered tip, bent at a right angle, was tied into the inferior thyroid vein. Blood samples were obtained from the thyroid vein in one of three ways. (1) Blood was allowed to flow freely from the thyroid vein cannula into a graduated centrifuge tube, the volume (usually 5–10 ml.) being measured every ¼ hr, 0·5 ml. removed, and the remainder

of the blood then returned to the animal through the saphenous artery cannula. (2) The thyroid vein cannula was joined to a further polythene cannula inserted via the anterior facial vein into the external jugular vein. The junction between the cannulae was broken every ½ hr to allow the collection of about 7 drops of blood. The flow of blood through the cannula system was observed with a low-powered ( × 40) microscope after painting the outside of the tube with oil. (3) The thyroid vein cannula was closed with a small polythene spigot which was removed at quarter-hour intervals for the collection of about 7 drops of blood. To avoid contamination of the sample with stagnant blood in the cannula and vein, the first 3 drops were discarded. The rate of flow of blood drops was measured with a stopwatch. 0·1–1·0 ml. of each sample was pipetted into a small glass container and diluted with water where necessary to bring the volume in all cases to 1·0 ml. Measurement of the radioactivity was made with the use of a well-type scintillation counter (Ekco Type N 550A), with an automatic scaler (Ekco Type N 530D). In the later part of the study a single-channel pulse analyser (I.D.L. Type 672) was also used. In some experiments samples of thyroid vein blood were analysed for their content of radioactive protein-bound iodine ($PB^{131}I$). The procedures used have been described by Brown-Grant, von Euler *et al.* (1954).

*Adrenalectomy* was performed as described by Brown-Grant, Harris & Reichlin (1954). During the first stage operation opportunity was taken of the wide exposure of the abdominal contents to remove any accessory nodules of adrenal tissue.

*Electrical stimulation of hypothalamus or pituitary gland* was performed by means of electrodes inserted 1–2 weeks before the experiment. The electrodes consisted of platinum or platinum-iridium wire (S.W.G. 34) insulated to within 0·5–1·0 mm of the tip with glass capillary tubing. The insertion of the electrodes has been described by Harris & Woods (1958). For bipolar stimulation two electrodes with their uninsulated tips 0·5–1·0 mm apart were used. For unipolar stimulation a single electrode was implanted in the head and the indifferent electrode placed in the mouth or rectum. At the beginning of the experiment a length of lead was extracted through a small nick in the scalp and soldered to leads from the stimulator. Stimulation was effected by means of sine-wave a.c., 50 c/s, at 1·0, 1·5 or 2·0 V. A mercury switch with rotating cam allowed stimulation to be applied during alternate quarter minutes for periods of 1–4 hr.

### *Drugs*

*Thyrotrophic hormone* was obtained from Armour and Co., Chicago, U.S.A. (Thyrotrophin, batch nos. R 377157 and R 3506), Organon Laboratories Ltd, (Ambinon, batch nos. 5984A and 6549), Ferring A. B., Malmö, Sweden (Actyron, batch no. 8041), and the National Institute for Medical Research, Mill Hill, London (international standard preparation). Solid preparations were dissolved in NaCl solution 0·9 % (w/v) and all were administered by intravenous or subcutaneous injection in doses ranging from 4 to 200 mg equivalent of U.S.P. standard.

*Adrenaline* (adrenaline acid tartrate, British Drug Houses Ltd, and adrenaline hydrochloride, Allen and Hanburys Ltd) was infused intravenously at rates of 1·4–8·0 μg/min (as base).

*Noradrenaline* (L-noradrenaline bitartrate, Bayer Products Ltd, batch no. JC 781) was injected I.V. in doses of 10–50 μg (as base).

*N-(phenoxyisopropyl)- N-benzyl-β-chlorethylamine hydrochloride* (Dibenyline, Smith Kline and French) was dissolved in propylene glycol and injected subcutaneously in doses of 1–10 mg/kg body wt.

11-*dehydro*-17-*hydroxycorticosterone* (cortisone acetate; Upjohn) was used to maintain the completely adrenalectomized rabbits.

### *Histology*

The histological procedures were those described previously by Harris & Woods (1958)

## RESULTS

### *The anatomy of, and blood flow through, the thyroid vessels in the rabbit*

Plate 1, figs. 1 and 2, illustrates the anatomy of the thyroid blood vessels in the rabbit, as determined by dissection after injection of neoprene latex into the vessels. The arterial supply to the thyroid enters the upper part of the gland. The venous drainage of the thyroid enters first a plexus of veins situated between the trachea and oesophagus. This plexus is drained (1) by the two inferior thyroid veins passing caudally, and (2) by wide communications to a plexus of veins on the thyrohyoid membrane, which in turn drains into the pterygoid venous plexus on either side and thus into the external jugular veins. It is important to note that the venous plexuses have large anastomotic veins crossing the mid line, so that a cannula in one of the four veins draining the venous plexuses around the larynx delivers blood from a general thyroid pool. The inferior thyroid veins are often joined by a small tributary coming directly from the region of the isthmus and lower pole of the gland. This tributary must be carefully avoided when inserting the cannula, for if it is inadvertently blocked a small region of the gland becomes obviously congested with blood with possible leakage of radioactivity into surrounding tissues. It is apparent that the inferior thyroid vein drains blood derived in large measure from the thyroid but to a small extent also from the trachea, oesophagus and other structures in the vicinity.

The average blood flow through the thyroid vessels of the rabbit has been given as about 6 g blood/min/g thyroid tissue (Monkus & Reineke, 1958). In the present study the thyroid weight was found to be 165·0 mg (S.E. $\pm$ 9·1) in a series of twenty-two normal adult female chinchilla rabbits. The average absolute blood flow through the thyroids of these animals would therefore be 1·0 ml./min. This figure is in general agreement with data given by Söderberg (1958).

The blood flow from a cannula placed in an inferior thyroid vein was about 0·3–0·6 ml./min. The possibility was considered that cannulation of an inferior thyroid vein might result in sufficient interference with the thyroid venous drainage to cause congestion, oedema and damage to the thyroid gland. Such a possibility is especially pertinent in the experiments in which the cannula was blocked with a spigot except when blood collections were made at quarter-hour intervals. However, the following observations indicate that such interference with the thyroid circulation did not occur: (1) The macroscopic appearance of the gland at the end of the experiment was normal in all cases. In twenty-one animals the thyroid was removed and fixed at the end of the experiment or 48 hr after ligation of an inferior vein, and the microscopic appearance found to be

normal. (2) The hydrostatic pressure in the cannula in the inferior thyroid vein was low, as demonstrated by the fact that raising the tip of the cannula slightly above the neck level resulted in slowing or stopping of blood flow. (3) The free exit of blood by other routes indicated by the immediate and marked (two or three times) increase in rate of blood flow from the cannula when the vessels on the thyrohyoid membrane were cauterized or ligated. The profuse venous drainage of the thyroid was also demonstrated by retrograde injection of Indian ink through the cannula in the inferior thyroid vein at the end of many experiments. The ink rapidly filled the laryngeal venous plexus and appeared in the external jugular veins.

*The viability of the experimental preparation*

One hundred and nine experiments have been performed. In twenty-six cases the animals died within 8 hr, and in twenty of these in less than 4 hr. The usual duration of an experiment was 8–10 hr. These deaths could not be explained by an overdose of anaesthetic, since anaesthesia was easily maintained at a light surgical level with intratracheal ether. Also the surgical trauma involved was slight and the body temperature of the rabbit carefully controlled with a rectal or vaginal thermometer. After the preliminary experiments the blood pressure was recorded at quarter-hour intervals and it was observed that in the rabbits that died a marked fall in blood pressure occurred in the ½–1 hr period preceding death. Intravenous injections or infusions of 0·9% sodium chloride or 'Dextraven' (6% (w/v) solution of dextran in 0·9% sodium chloride; Benger Laboratories Ltd) resulted in only transient and slight improvement in these cases. In the later stages of the work review of the deaths showed that twenty-three out of twenty-six occurred in experiments in which shed blood had been returned to the animal. This was necessary when blood from the cannulated thyroid vein had been allowed to flow freely throughout the experiment either into a graduated tube or through the polythene anastomosis to the external jugular vein. In forty-eight experiments in which the cannula had been blocked by a spigot and only 0·1 ml. blood samples collected at quarter-hour intervals, no shed blood was returned to the animal, and only three animals died throughout the experiment. This technique was used in all later experiments.

*Comparison of radioactivity in thyroid vein (V) and arterial blood (A) during the control period*

The general plan of the experiments was as follows. After insertion of cannulae in the saphenous arteries and thyroid veins, blood samples were collected from the thyroid vein, and the blood pressure and body tempera-

ture recorded at quarter-hour intervals. Arterial samples were taken at ¼–2 hr intervals, since the radioactive content of these samples changed slowly. After ½–6 hr (usually 1–2 hr) of control observations, an injection of thyrotrophic hormone was given or electrical stimulation of the hypothalamus was applied, and observations were continued.

Ten measurements made on the blood of six rabbits, both before and after administration of thyrotrophic hormone, showed that the plasma protein-bound $^{131}I$ varied from 77·7 to 103·0 % (average 90·6 %) of the total plasma $^{131}I$. These observations were made 2–4 days after the administration of 20–40 $\mu$c $^{131}I$, and are in agreement with the findings of Brown-Grant (1955). It seems therefore safe to conclude that any significant change in the radioactive content of the blood in the present experiments reflects a change in concentration of plasma $PB^{131}I$, that is, a change in thyroid hormone concentration.

Comparison was made of the radioactivity in the first thyroid-vein blood sample ($V$) with that in arterial blood ($A$) collected simultaneously. These samples were obtained 1–2 hr after the beginning of anaesthesia and operative procedures. In fifty-three animals the thyroid-vein blood had a radioactive content equal to, or slightly higher than, that of arterial blood ($V/A$ = 100–120 %). In twenty-seven animals the thyroid vein blood had 120–200 % the activity in arterial blood, and in six animals 200–500 %. It was a frequent finding that in animals, in which a large $V$-$A$ difference was observed in the first blood samples, this difference decreased often to zero within 1–2 hr (see Text-fig. 2). It is probable that the stress of anaesthesia and operations resulted in depression of secretion of thyrotrophic hormone and inhibition of thyroid activity (see Brown-Grant, von Euler *et al.* 1954).

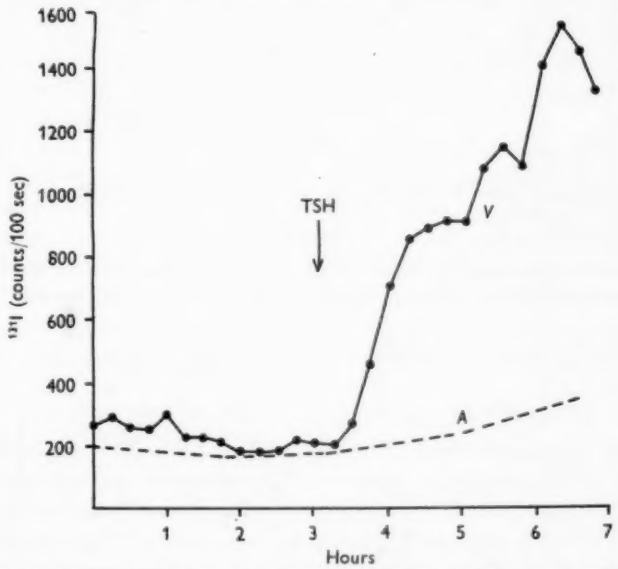
In six out of one hundred and seven animals an increase in the thyroid-vein $^{131}I$ content was observed during the control period. These rises occurred in an apparently spontaneous fashion. It is possible that they reflect renewed release of thyrotrophic hormone from the pituitary gland, or that they were due to undetected damage to the thyroid gland or its circulation.

### *The effect of injection of thyrotrophic hormone (TSH)*

The effect of injection of TSH was studied in forty rabbits. Four different thyrotrophic preparations were injected intravenously, in doses ranging from 4 to 200 mg equivalent U.S.P. standard powder. In view of the fact that adrenaline or sympathetic-induced vaso-constriction of the thyroid blood vessels might influence the results, studies were made on both normal rabbits (12), and rabbits previously given a sympatholytic agent (Dibenyline) (20) or subjected to complete adrenalectomy (8). The

Dibenyline was administered subcutaneously the night before, and on the morning of, the experiment in doses of 5–10 mg/kg body wt. The adrenalectomized animals were maintained on small doses of cortisone. No differences were observed between the responses of these and the normal animals to injection of TSH.

The typical response to injection of TSH is shown in Text-figs. 1, 2 and 3. After a latent period of 15–30 min the radioactivity in thyroid vein blood increased. The peak of the response occurred between 1 and 3 hr after injection (average 115 min) and showed a rise to $1\frac{1}{2}$–9 times (average 3·8 times that of the initial value of arterial blood). Following the peak of

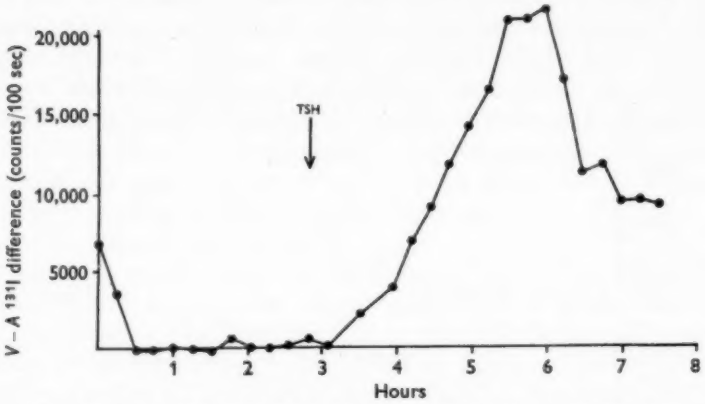



Text-fig. 1. Rabbit 1256. Thyroid response to injection of TSH at arrow. *V*, radioactivity of thyroid vein blood; *A*, radioactivity of arterial blood.

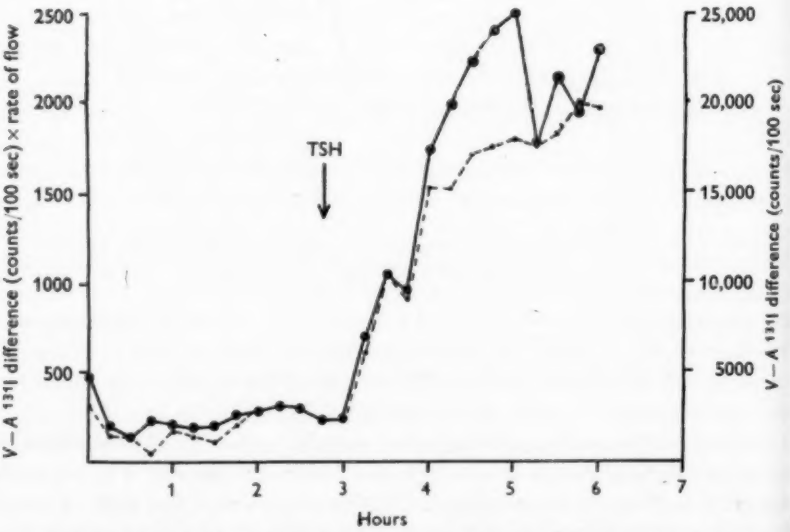
the response, the radioactivity in the thyroid vein blood fell gradually and was usually still high several hours later. The radioactive content of arterial blood usually showed changes similar to that of the thyroid vein blood but on a greatly reduced scale (see Text-fig. 1).

The rate of blood flow through the cannula in the thyroid vein showed some variation throughout the course of any experiment. The example depicted in Text-fig. 1 is typical. In this experiment the flow of blood from the thyroid vein varied from 3·7 to 9·5 ml./15 min over the 7-hr period. However, the curves representing the results have the same general shape whether the radioactivity of thyroid vein blood per unit

volume or unit time of collection is plotted against time (Text-fig. 3). This indicates that the variations in thyroid blood flow which occurred do not affect the general interpretation of the results in terms of thyroid activity. This may be due in large measure to the fact that at the time of injection of TSH the release of thyroid hormone had almost completely ceased




Text-fig. 2. Rabbit 1249. Thyroid response to injection of TSH. Difference in radioactivity between thyroid vein blood and arterial blood plotted against time.




Text-fig. 3. Rabbit 1259. Thyroid response to injection of TSH plotted as in Text-fig. 2 ●—●, and also as the product of the arterio-venous radioactivity difference and the rate of thyroid vein blood flow ●– – –●.

(Text-figs. 1, 2, 3). Under these circumstances the hormone content of the venous blood leaving the gland would not be appreciably affected by changes in rate of blood flow. The fact that the $PB^{131}I$ content of arterial blood showed increases of up to double the initial value emphasized the great increase in thyroid secretion which occurred.

*The effect of adrenaline and noradrenaline*

Since it has been reported that injection or infusion of adrenaline or noradrenaline affects thyroid activity, it was of interest to study the effect of these drugs under the conditions of the present experiments. Single or multiple injections of L-noradrenaline bitartrate (10–50 $\mu$g doses, as base) were given intravenously to seven rabbits. In four cases the injection followed administration of TSH. In no case did the noradrenaline appear to affect the *V-A* difference, either when given alone or given at the peak of a thyroid response to TSH.

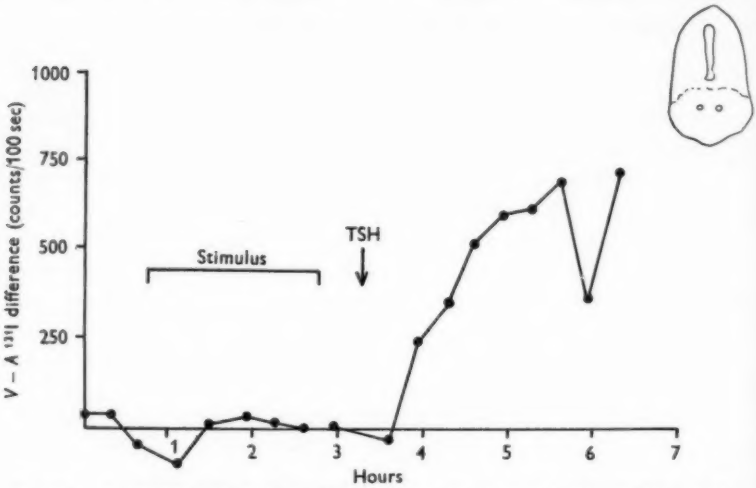
Adrenaline acid tartrate or hydrochloride infusions were given intravenously to five rabbits at a rate of 1·4–8·0 $\mu$g/min (total dose of 116–1100 $\mu$g, as base) for periods of 72–110 min. In two animals the infusions were started 14 and 43 min before injection of TSH and in the other three the infusions were given during the peak of a thyroid response to TSH injection. In these few cases no increase of thyroid vein blood radioactivity could be ascribed to the adrenaline infusion, and no obvious change was observed in the response to injection of TSH.

*The effect of electrical stimulation of the hypothalamus*

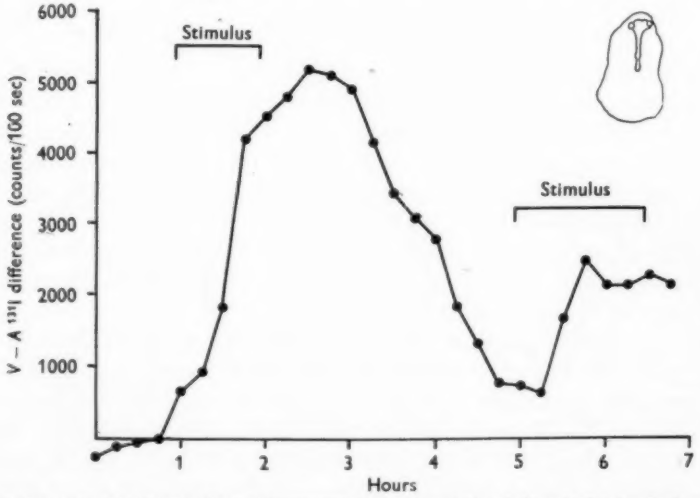
The effect of electrical stimulation of the hypothalamus was studied in twenty-eight rabbits. Fourteen of these were pre-treated with Dibenyline, three were subjected to adrenalectomy and eleven were normal animals. No distinction could be made between these three groups on the basis of their thyroidal response to electrical stimulation. Thirty-three periods of stimulation, lasting for $\frac{3}{4}$–6 hr (usually 1–2 hr), were applied in these animals. If no thyroidal response occurred during or after the period of stimulation, the reactivity of the thyroid gland was controlled by observing the response to injection of TSH.

Electrical stimulation of various regions of the hypothalamus or pituitary gland resulted in no observable change in thyroid activity in twelve animals, and an increase in sixteen animals. In the latter sixteen animals the responses were qualitatively similar to those observed after injection of TSH (Text-figs. 4, 5 and 6). A latent period of 15–30 min was followed by a rise of radioactivity in the thyroid vein blood which reached a peak within $\frac{1}{2}$–3 hr (average 90 min) after starting stimulation. The magnitude of the peak radioactivity varied from 1·3 to 4·7 times (average 2·2 times)

that of the initial value in arterial blood. In eight cases the peak level of the *V-A* difference was maintained throughout the period of stimulation; in the other eight animals the response diminished though stimulation was

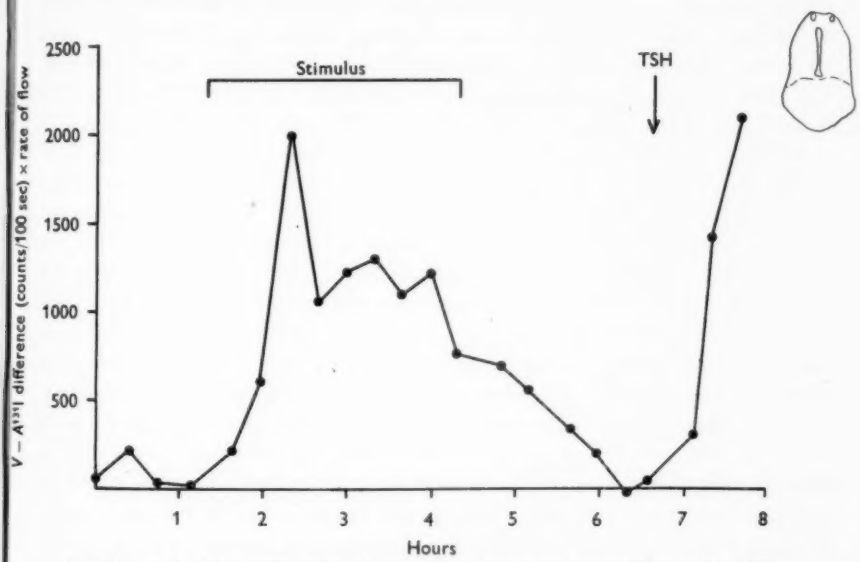



Text-fig. 4. Rabbit 1209. Thyroid responses to electrical stimulation of the hypothalamus, followed by injection of TSH. Inset, outline of horizontal section of the hypothalamus, showing the position of the electrode tips (see Pl. 2, fig. 8).




Text-fig. 5. Rabbit 1216. Thyroid response to two periods of electrical stimulation of the hypothalamus. Inset, outline of horizontal section of hypothalamus, showing the position of the electrode tips (see Pl. 2, fig. 4).

maintained. In all animals the *V-A* difference had approximated to previous control values within 3 hr of the end of stimulation. Repetition of the stimulus was followed by a second response, which was however smaller than the first (see Text-fig. 5). In ten animals a small but distinct rise in radioactivity in arterial blood was observed in relation to the thyroidal response.




Text-fig. 6. Rabbit 1208. Thyroid response to electrical stimulation of the hypothalamus followed by injection of TSH. Plotted as the product of the arteriovenous radioactivity difference and the rate of thyroid vein blood flow. Inset, outline of horizontal section of hypothalamus, showing position of the electrode tips (see Pl. 2, fig. 3).

The site of the stimulating tips of the electrodes was determined histologically from study of serial sections through the region of the hypothalamus and pituitary gland (Pl. 2). In all sixteen animals in which a thyroid response had been obtained the electrode was found to be in a position to stimulate the supraopticohypophysial tract, either in the median eminence or in the tuber cinereum anterior to the median eminence. In the animals in which a thyroid response had not been obtained the electrodes were found to be in the mammillary region of the hypothalamus (3 rabbits), mid-line tuberal region of the hypothalamus (5 rabbits), anterior pituitary gland (1 rabbit) and the region of the supraopticohypophysial tract (3 rabbits).

## DISCUSSION

Protein-bound $^{131}I$ has been found to account for 90 % of the total plasma radioactivity 30 hr after the injection of radioactive iodine into female rabbits (Brown-Grant, 1955). In the present study 77·7–103·0 % (average 90·6 %) of the plasma radioactivity was protein-bound 2–4 days after administration of $^{131}I$. The animals used were kept under similar conditions in the same laboratory as those investigated by Brown-Grant (1955) and the results are in good agreement. It is likely that, for technical reasons, the above average is somewhat lower than the true figure. There can be little doubt that changes in radioactivity of the blood, under the present conditions, reflect changes in blood concentration of thyroid hormone.

The significance of the function of an endocrine gland for the organism as a whole is most clearly expressed in terms of quantity of hormone secreted per unit time. This quantity may be defined in terms of the rate of blood flow through the gland and the hormonal concentrations in the arterial blood entering, and the venous blood leaving, the gland. In the present experiments the concentration of hormone in arterial blood was accurately measured and the concentration in thyroid vein blood, which is slightly diluted with blood from the larynx, trachea and oesophagus, was also obtained. The absolute rate of blood flow through the thyroid was not measured. One of four veins draining the thyroid region was cannulated and the rate of flow through this vein observed throughout the experiments. In general it was assumed that the flow of blood observed in the inferior thyroid vein is related to total thyroid blood flow. However, two factors have to be considered. First, constriction of the inferior thyroid vein or clotting of blood around the mouth of the cannula in the vein (which seemed to occur early in the experiments, perhaps owing to the manipulations during cannulation) results in a reduction in the rate of flow observed, which is not necessarily related to a reduction in the rate of total thyroid blood flow. Under these circumstances the concentration of radioactivity in thyroid-vein blood remains constant, though the total amount of radioactivity discharged through the inferior thyroid vein per quarter hour falls. Secondly, vasoconstriction of the thyroid vessels in the gland itself results also in a reduction of rate of flow through the cannulated vein. This seemed to occur more frequently late in the experiments, possibly as a consequence of a fall in the general systemic blood pressure. This results in a proportionate rise in the concentration of radioactivity in thyroid vein blood although the amount of hormone secreted per quarter hour, and the amount of hormone collected from the cannulated thyroid vein per quarter hour, remain constant. The results may be expressed graphically in one of two ways. Either the difference in

concentration of radioactivity in arterial and thyroid vein blood (*V-A*) may be plotted against time, or the product of the *V-A* difference and the rate of flow from the inferior thyroid vein (*V-A* × rate of flow = total radioactivity liberated by the thyroid into the inferior thyroid vein) may be plotted against time. Text-figure 3 shows the result of a typical experiment plotted in both ways. It may be seen that plotting *V-A* against time gives a smooth curve in the early control period, whereas *V-A* × flow rate plotted against time results in a smooth curve toward the end of the experiment. The general features of the response plotted in the two ways differ little.

The proportion of thyroid venous blood collected from a cannulated inferior thyroid vein is probably about 50 %. The most accurate data on thyroid blood flow in the rabbit appear to be those of Monkus & Reineke (1958) and Söderberg (1958). For a thyroid gland of 165 mg weight the blood flow would be about 1 ml./min. In the present experiments an average collection rate was ½ ml./min. This figure does not necessarily represent the normal rate of blood flow in the inferior thyroid vein, since it is dependent on the hydrostatics of the cannula system. The data mentioned above on which the estimate of thyroid blood flow is based, and our own data regarding the proportion of thyroid venous blood collected from the cannulated vein, include an error due to blood from tissues other than the thyroid draining into the inferior thyroid vein. This error has not been evaluated but is probably small.

The present procedure as applied to the thyroid gland has a great advantage as compared with similar experiments on the adrenal gland. The thyroid gland of the rabbit is rapidly inhibited by the stress of anaesthesia and surgical trauma (Brown-Grant, von Euler *et al.* 1954). Thus the effect of various procedures in evoking secretion of TSH can be observed against a quiescent background. The reverse is the case with the adrenal gland in which the non-specific stimuli associated with the preparation of the animal result in marked adrenal activation. In the present work it was frequently observed that a large *V-A* difference in the first blood samples rapidly decreased or disappeared. Similar observations have been made by Söderberg (1958). This affords evidence of the inhibitory effect of stress on rabbit thyroid function.

It is difficult to convert results obtained with $^{131}I$ into absolute terms of thyroid function. If accurate figures were available for the blood concentrations in an animal of $PB^{127}I$ and $PB^{131}I$ at any given moment, then, on the assumption that the $PB^{127}I$ and $PB^{131}I$ were in equilibrium, values for radioactivity could be converted into micrograms of thyroxine. Such figures were not available in the present work. It is not possible to express any given response of the thyroid as a proportion of its normal level of activity on the basis of radioactive measurements alone, since by the time

the first sample is collected the normal activity of the thyroid has been inhibited by the procedures involved. Therefore a thyroid response in an animal cannot be compared quantitatively with that of another, though the magnitude of multiple responses in a single animal may be so compared.

The response of the thyroid gland to an injection of TSH occurs more rapidly than was suspected a few years ago, since it becomes apparent in 15–30 min. The figure for this latent period in rabbits, given by Söderberg (1958), of 5–30 min is probably more exact owing to the method used for collecting blood samples. Ackerman & Arons (1958) report a latent period of 15-20 min in the dog. Comparison may also be made with the time relations (latent period about 3 hr) of the thyroid inhibition which follows injection of thyroxine or application of a stressful stimulus (Brown-Grant, von Euler *et al.* 1954; Brown-Grant, Harris & Reichlin, 1954). Söderberg (1958) reports a cessation of thyroid activity within 3 hr of the beginning of operative trauma and a similar figure has been found in the present study.

Administration of adrenaline or noradrenaline has been reported to influence thyroid activity. Adrenaline was found to be active in causing a constriction of the thyroid blood vessels (Gunning, 1917; Mason, Markowitz & Mann, 1930) and a reduction in thyroid blood flow (Söderberg, 1958). Brown-Grant & Gibson (1956) reported that intravenous infusion of 2–4 $\mu$g/min of adrenaline bitartrate produced a complete inhibition of the uptake of $^{131}$I by the thyroid of the rabbit. In view of the above findings it is surprising that Ackerman & Arons (1958) report that adrenaline infusion in dogs results in a marked increase in secretion of thyroid hormone. Söderberg (1958) found that small doses of adrenaline or noradrenaline in rabbits and cats pre-treated with TSH evoke a transient (about 1 min) increase in thyroid secretion, though large doses of these hormones were inhibiting. In the present study intravenous infusion of adrenaline (1) did not abolish, nor alter qualitatively, the thyroid response to a subsequent injection of TSH, and (2) had no obvious effect on a thyroid response to a previous dose of TSH.

Electrical stimulation of the hypothalamus resulted in a thyroidal response similar in its time relations to that following administration of TSH. The magnitude of the responses seemed less than those seen after injection of large doses ($>$ 4 mg equivalent U.S.P. standard) of TSH. This observation is based on experiments on animals that had responded to electrical stimulation of the hypothalamus with increased thyroidal activity and in which a subsequent injection of TSH evoked a similar but greater response. It is noteworthy that electrical stimulation of the hypothalamus induced an increase in thyroid activity even in the presence of a stress-induced thyroidal inhibition. Since Harris & Woods (1958)

have noted a maintained increase in thyroid function, consequent to hypothalamic stimulation, in spite of a raised blood thyroxine level, it would seem that neural influences on the rate of TSH secretion preponderate over the inhibitory effects of stress and a raised thyroid-hormone concentration in the blood.

The hypothalamic site of electrical stimulation effective in exciting an increased release of TSH was in the region of the supraopticohypophysial tract. This confirms the previous observations of Harris & Woods (1958). Similar stimuli applied to other areas in the tuber cinereum, or to the mammillary bodies, or pituitary gland directly, failed to elicit any thyroidal response. It should be pointed out, however, that three out of twelve animals, in which stimulation was not followed by increased thyroid activity, were later found to have electrodes in the supraopticohypophysial region. The negative results in these animals may be attributed to one or more of many factors such as respiratory or circulatory failure. The finding that it is the supraopticohypophysial tract region which is the effective site of electrical stimulation agrees well with the reports of many workers (Ganong, Fredrickson & Hume, 1955; Greer & Erwin, 1956; Bogdanove, 1957; D'Angelo, 1958; Purves, Sirett & Averill, 1958) that lesions in the basal areas of the anterior hypothalamus are associated with decreased thyroid activity. Harris & Woods (1958) found that electrical stimulation of the supraopticohypophysial tract area in normal rabbits was followed by increased thyroid function. After adrenalectomy and maintenance on small daily doses of cortisone, the thyroidal response to stimulation was increased. In six other animals, in which the electrodes were situated slightly more posteriorly in the hypothalamus, and in which no thyroid response had been obtained previous to adrenalectomy, constant and marked thyroidal activation in response to stimulation was observed after this operation. In the present work experiments could not be done in the same animal before and after adrenalectomy. However, the response obtained in adrenalectomized animals did not show any obvious differences from those obtained in other rabbits. It is difficult to draw any conclusions in this respect since the periods during which thyroid function was investigated differed so greatly in the two studies. The fact that adrenalectomized rabbits and those treated with Dibenyline show a clear-cut response to hypothalamic stimulation demonstrates that the effect of such stimulation is independent of activation of the adrenal medulla or sympathetic system.

## SUMMARY

1. A technique has been devised for observing acute changes in thyroid activity in anaesthetized rabbits over periods of about 8 hr. The animals, injected with $^{131}I$ 4 days previously, had cannulae placed in an inferior

thyroid vein and a saphenous artery, and blood samples were removed every quarter hour. About 90% of the radioactivity was found to be protein-bound, so that changes in radioactivity in arterial and thyroid vein blood could be taken to reflect changes in thyroid function. This preparation was used to observe the effects of administration of thyrotrophic hormone (TSH) and of electrical stimulation of different regions in the hypothalamus and pituitary gland. Normal and adrenalectomized rabbits, and rabbits pre-treated with Dibenyline, showed no differences in the thyroidal responses to such stimuli.

2. Control experiments showed that, under the conditions of the experiment, thyroid function rapidly decreases or disappears. This low-level activity persists for many hours and is probably due to the stress of anaesthesia and surgical trauma inhibiting TSH secretion by the pituitary gland.

3. Administration of exogenous TSH resulted in a marked increase in radioactivity in thyroid vein blood, commencing in 15–30 min and reaching peak values in about 2 hr. Injection or infusion of adrenaline or noradrenaline did not evoke such an increase, and did not modify the response to TSH.

4. Electrical stimulation of the hypothalamus in the region of the supraopticohypophysial tract resulted in changes in sixteen out of nineteen animals similar to those following injection of TSH. Stimulation in other regions of the hypothalamus or in the pituitary gland was without effect.

5. The rapid increase in radioactivity in thyroid vein blood, induced by injection of TSH or hypothalamic stimulation, was reflected by a small and slow rise in arterial blood radioactivity.

6. It is concluded that an acute preparation may be satisfactorily used for investigation of factors influencing thyroid function; that the thyroid responds rapidly to an increased concentration of TSH in the blood; and that electrical stimulation of the region of the supraopticohypophysial tract of the hypothalamus causes a discharge of TSH from the anterior pituitary gland.

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## EXPLANATION OF PLATES

### PLATE 1

Anatomy of the thyroid blood vessels in the rabbit. Fig. 1. The thyroid arteries. (Dissection following injection of the arterial system with neoprene latex). Fig. 2. The thyroid veins (Neoprene latex cast).

### PLATE 2

Photomicrographs of horizontal sections through the hypothalamus and surrounding structures in different animals. The sections are 100 $\mu$ thick and are stained with haematoxylin and eosin or Weigert's iron-haematoxylin. A, anterior wall of thyroid ventricle; E, site of uninsulated tips of electrodes; IC, internal carotid artery; MB, mammillary body; V, third ventricle. Fig. 1. Rabbit 1208. Bipolar electrodes in anterior tuber cinereum; thyroid excitation on stimulation (see Text-fig. 6). Fig. 2. Rabbit 1216. Bipolar electrodes in median eminence; thyroid excitation on stimulation (see Text-fig. 5). Fig. 3. Rabbit 1284. Bipolar electrodes in anterior tuber cinereum; thyroid excitation on stimulation. Fig. 4. Rabbit 1190. Bipolar electrodes in median eminence; thyroid excitation on stimulation. Fig. 5. Rabbit 1197. Bipolar electrodes in tuber cinereum, posterior to region of supra-opticohypophysial tract; no thyroid excitation on stimulation. Fig. 6. Rabbit 1209. Bipolar electrodes in mammillary body; no thyroid excitation on stimulation (see Text-fig. 4).

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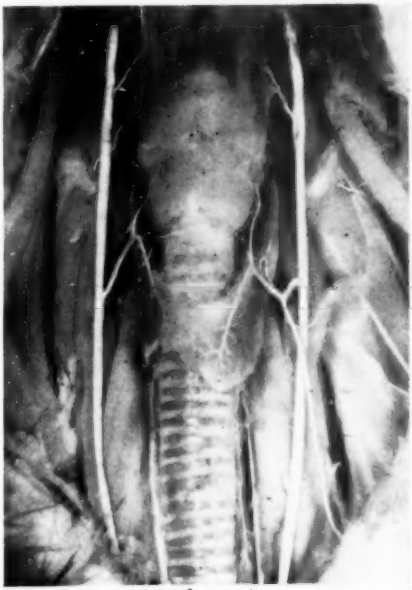


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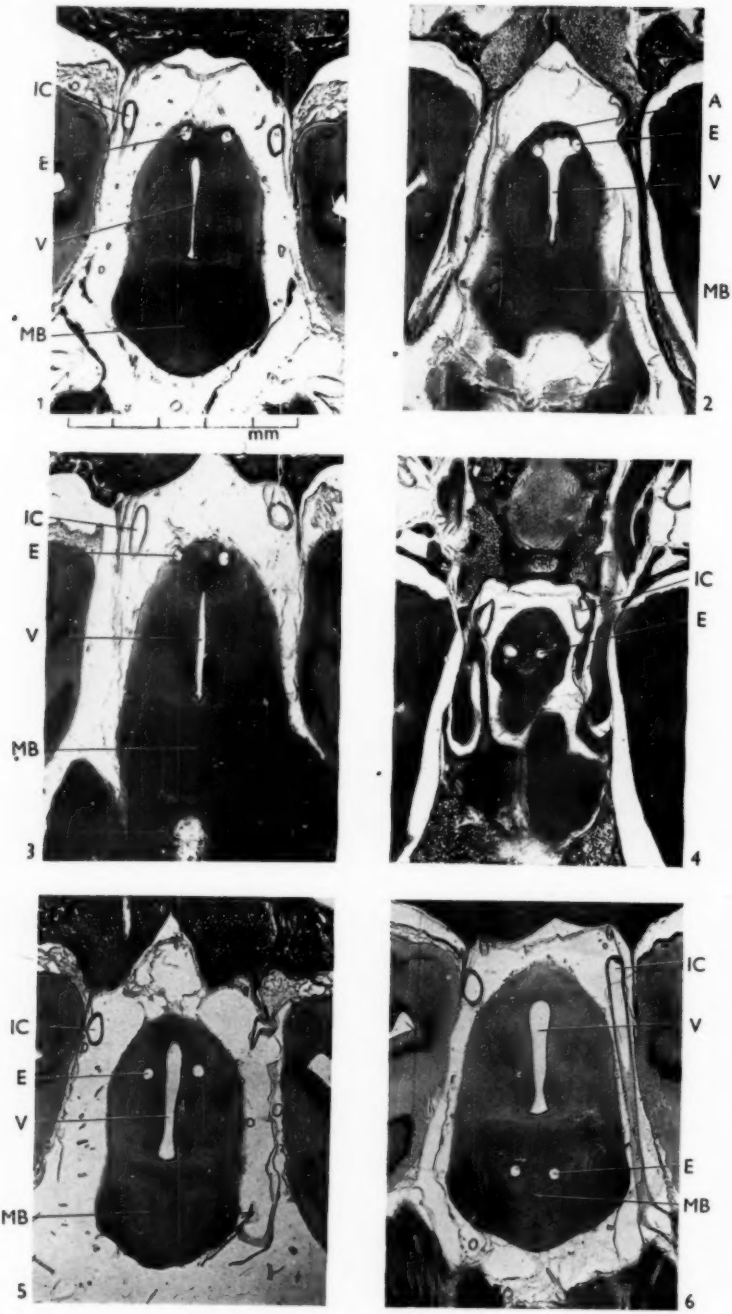


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# THE PHOSPHORUS METABOLISM OF SQUID AXONS AND ITS RELATIONSHIP TO THE ACTIVE TRANSPORT OF SODIUM

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The work described in this paper was undertaken as a result of the finding of Hodgkin & Keynes (1955) that the sodium efflux from the giant axons of cephalopods can be markedly reduced by the action of metabolic inhibitors. Hodgkin and Keynes showed that the efflux of $^{24}Na$ from the giant axons of the squid *Loligo forbesi* and the cuttlefish *Sepia officinalis* was reduced when the axons were immersed in artificial sea water containing cyanide, 2.4-dinitrophenol (DNP) or azide. If at a later stage the axons were immersed in inhibitor-free artificial sea water there was a substantial recovery of the efflux. In experiments with *Sepia* axons the changes in the $^{24}Na$ efflux were found to correspond with changes in the $^{42}K$ influx, which decreased in the presence of the inhibitors and increased again when the inhibitors were removed. The $^{24}Na$ influx and the $^{42}K$ efflux were not, on the other hand, affected to a significant extent by the inhibitors. The effects of metabolic inhibitors on the sodium efflux from the giant axons of the squid *Loligo pealii* have been studied by Shanes & Berman (1955) and it was shown that anoxia causes a decrease in the efflux of $^{22}Na$. Shanes and Berman also treated some axons with monoiodoacetate and their results suggest that this inhibitor does not affect the sodium efflux. The effects of ouabain on the $^{22}Na$ efflux from the giant axons of *Loligo forbesi* have been studied (Caldwell & Keynes, 1959) and this substance has been found to cause a sharp fall in the efflux.

Various suggestions have been made about the way in which the energy required for the active transport of ions across cell membranes may be derived from metabolism. The most notable of these are the Redox Pump theory of Conway (see, for example, Conway, 1958) and the idea that the transport of ions may be linked in some way to the phosphate esters in the cell, in particular to those with 'high-energy' phosphate bonds. Since metabolic inhibitors had been found to inhibit the efflux of sodium and the influx of potassium of cephalopod axons it seemed that an investigation of their effects on the phosphorus metabolism might provide evidence for a

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correlation between the ability of the axons to transport sodium and potassium and the content of 'high-energy' phosphate esters. Such a correlation has in fact been found and it therefore seems likely that the 'high-energy' phosphate esters play some part in the mechanism of ion transport.

Preliminary accounts of various parts of this work have been given to the Physiological Society (Caldwell, 1956), to the Biochemical Society (Caldwell, 1957), to a colloquium organized by the Institut National des Sciences et Techniques Nucléaires (Caldwell, 1958*a*) and to the 4th International Congress of Biochemistry (Caldwell, 1958*b*).

## METHODS

*Material.* The hindmost stellar giant axon of *Loligo forbesi* was used in the whole of this work. Whenever possible the giant axons from large specimens (mantle length up to 20 in., 50 cm), which are available between October and January, were used. The hindmost giant axons from these specimens are usually 700–900 $\mu$ in diameter and chromatographic separation and estimation of the phosphate compounds can be carried out on extracts from only one axon. In a few cases, when large squid were not available, pairs of the hindmost stellar giant axons from two or three smaller squid were used, one axon from each pair being exposed to one set of conditions and the other axon to the contrasting set of conditions. When more than one pair of axons was used the axoplasm from each set of axons which had been exposed to a given set of conditions was pooled.

*General experimental procedure.* In all experiments the axons were first carefully dissected from the mantle and then tied at both ends with cotton. It was not found necessary to clean away the rest of the nerve trunk in order to carry out the experiments. After dissection the axons were made to conduct impulses at the rate of about 50/sec for 5–10 min, since the axons used by Hodgkin & Keynes (1955) had been subjected to this treatment in order to load them with sodium. The axons were allowed to recover for a short while in sea water and then the experiment was started.

The experiments were usually carried out in one of two ways. In one type of experiment one axon of a pair was soaked in a 'sea water' containing an inhibitor while the other was soaked in inhibitor-free sea water. After a given period the two axons were taken for analysis of the phosphate compounds. In the second type of experiment the axons were soaked in a 'sea water' containing an inhibitor, usually for 90–105 min, and then one axon of a pair was taken for analysis of the phosphate compounds while the other was transferred to inhibitor-free sea water. After 50–75 min in inhibitor-free sea water the second axon was taken for analysis of the phosphate compounds.

The following inhibitors and inhibitor concentrations were used: cyanide, 2 mM; DNP, 0·2 mM; azide, 3 mM; ouabain, $10^{-3}$ M. These cyanide, DNP and azide concentrations fall within the concentration ranges used by Hodgkin & Keynes (1955) to inhibit the sodium efflux from *Sepia* and *Loligo* giant axons, while the ouabain concentration is that used by Caldwell & Keynes (1959).

The ability of the axons to conduct action potentials was checked at various stages during the experiments, in particular just before each axon was taken for analysis of the phosphate compounds. Axons or parts of axons which were no longer excitable were discarded. The temperature during the experiments was usually between 14 and 20° C., pH measurements were done with a glass electrode system and a pH meter.

*Separation and analysis of the phosphate fractions.* The axoplasm was first extruded from the axon. This was usually done in a cold room. The axon was first placed on a cooled surface in order to cool it. After about a minute it was blotted with a piece of filter paper in order

to remove the adhering moisture and it was then cut at the ganglion end. The axon was then placed on a cooled microscope slide and the axoplasm pressed from the cut ganglion end on to the slide by means of a glass rod. The axoplasm was then rapidly transferred into a small glass cup containing a weighed amount ($W_1$, usually 50–150 mg) of cold 5% trichloroacetic acid (TCA) and the lumps of axoplasm were squeezed and pressed with a small glass rod for a few minutes to facilitate the penetration of the acid. The cup and its contents were then weighed to obtain the weight of the axoplasm ($W_2$) from the increase in weight. ($W_2$ was usually 25–60 mg; the weight of material lost on the glass rod used for squeezing the axoplasm was found to be negligible.) The cup and its contents were then left in a refrigerator for about an hour to allow the extraction of the phosphate compounds to take place. The lumps of axoplasm were then squeezed once more and as much as possible of the TCA extract was removed with a small glass pipette and transferred to a second small weighed glass cup. The weight ($W_3$, usually 55–105 mg) of the extract transferred to the second cup was then obtained from the increase in weight. At no stage in the extraction process did the denatured protein material of the axoplasm show any tendency to disperse in the TCA and it was quite easy to remove the extract from this material without any preliminary separation procedure such as centrifuging.

A small quantity ($W_4$, usually 15–30 mg) of an approximately 0·1 M solution of di-sodium ethylene-diamine-tetra-acetate (EDTA) in concentrated ammonia was then added to neutralize the TCA and to bind the calcium and magnesium in the extract in the form of soluble complexes and so prevent them from interfering with the chromatography of the phosphate compounds. As much as possible of the neutralized extract was then transferred to a second small glass pipette which was then weighed. Successive quantities of the extract were then applied to the filter paper for the chromatographic separation. The extract was confined to an area of about 1–2 $cm^2$ and after each application the moisture was removed by blowing a current of cold air over the paper. When all the extract had been put on the paper, the weight applied ($W_5$, usually 70–130 mg) was obtained by weighing the small glass pipette once more. The chromatogram was then run in two dimensions in the way described previously for muscle extracts (Caldwell, 1953), being run in one dimension for about 15 hr in an *n*-propanol–ammonia solvent (60 ml. *n*-propanol–30 ml. conc. ammonia–10 ml. distilled water) and for about 6–10 hr (i.e. until the solvent front had nearly reached the bottom of the paper) in the second dimension at right angles in a picric acid–tert-butanol solvent (80 ml. tert-butanol–20 ml. of a solution of picric acid (200 g/l.) in distilled water). In a few of the experiments the second dimension was run in a tert-butanol–TCA solvent (80 ml. tert-butanol–20 ml. of a solution of TCA (200 g/l.) in distilled water). The pattern of separation in this solvent is on the whole similar to that in tert-butanol–picric acid, the $R_F$ values being somewhat smaller. In one experiment the chromatogram was first run to the bottom of the paper in *n*-propanol–ammonia and after drying was then run to the bottom in the same direction in a slightly modified tert-butanol–TCA solvent (75 ml. tert-butanol–25 ml. of a solution of TCA (160 gm/l.) in distilled water).

The phosphate compounds were detected on the chromatograms by means of the molybdate–$H_2S$ method of Hanes & Isherwood (1949). In the case of chromatograms for which tert-butanol–TCA had been used as the second solvent the chromatogram was sprayed first with potassium permanganate (~ 70 mg/100 ml.) and put into an atmosphere of chlorine. According to Gerlach & Döring (1955), this treatment shows the presence of compounds containing adenine, in particular the adenosine phosphates, ATP, ADP and AMP, which appear as yellow spots which change to a deep red when subjected to alkaline conditions.

For the quantitative determinations of the phosphorus present in the various phosphate fractions the appropriate area of paper was cut out, oxidized with $H_2SO_4$ and $KHSO_4$, and the phosphorus then estimated colorimetrically. The procedure used was similar to that used in previous work on muscle (Caldwell, 1953). An improvement was introduced into the method in that the excess sulphuric acid was removed from the melt of $KHSO_4$ when

the oxidation was complete by sucking the sulphuric acid vapour out of the conical Pyrex digestion flask by means of a glass tube attached to a water pump. In this way the excess sulphuric acid was easily removed and very little of the vapour escaped into the fume cupboard and room.

The value (in $\mu$g P) obtained for the phosphorus in each of the phosphate fractions was multiplied by the factor $1000 \times (W_1 + W_2) \times (W_3 + W_4)/W_2 W_3 W_5$ to give the amount present in terms of $\mu$g P/g axoplasm. (This method of calculation assumes that at the stage of the TCA extraction the phosphate compounds are uniformly distributed between the TCA extract and the denatured insoluble axon debris. This is probably not so. The errors introduced were, however, probably small since the amount of insoluble debris was small in relation to the volume of TCA extract.)

In some of the experiments the lumps of insoluble axon debris remaining after the removal of the TCA extract were extracted a further two times with the TCA solution and then the total non-extractable phosphorus was determined by the method used for the determination of the phosphorus in the spots on the chromatograms.

It is unlikely that much of the arginine phosphate in the axons broke down during the course of the early stages of the analyses. A test in which a sample of arginine phosphate was subjected to the same treatments as the axoplasm as far as the stage just before the application of the extract to the paper showed that not more than 2% of the arginine phosphate breaks down during this part of the procedure. It is possible that there may be some slight break-down of the arginine phosphate during the running of the chromatograms in the second solvent, since a faint streak is sometimes visible below the arginine phosphate spot. However, in the course of this work one of the axons used was found to contain 161 $\mu$g P/g arginine phosphate and only 66 $\mu$g P/g orthophosphate. The maximum possible arginine phosphate break-down is therefore about 30%.

*DNP determinations.* Some experiments were done in which the amounts of DNP taken up by the axons from sea waters of different pH were measured. After soaking in the DNP-containing sea water, each axon was first washed rapidly in DNP-free sea water and dried quickly on the outside with a filter paper, and then the axoplasm was extruded. The axoplasm was put into a weighed glass cup containing 0·1 ml. of 5% TCA and the cup and its contents were reweighed to obtain the weight of the axoplasm. About 0·5 ml. of distilled water was added and the axoplasm was extracted during the night. The insoluble axon debris was then removed and 0·1 ml. of a 0·1 M solution of EDTA in ammonia was added to the extract. The ammonia converted the DNP into the yellow anionic form while the EDTA prevented the precipitation of any calcium or magnesium which might have been present. The extract was then made up to 1·3 ml. with distilled water. Absorption curves for the extracts were measured between 3500 and 5000 Å with a Unicam spectrophotometer. Extracts of axons which contained appreciable amounts of DNP gave curves very similar to those obtained with DNP standards containing the same amounts of TCA and EDTA as the extracts. A plot of (Optical density at 3900 Å − Optical density at 5000 Å) against the amount of DNP present was linear for the DNP standards and this plot was used as a calibration curve for the determination of the DNP contents of the extracts from the optical density readings at these two wave-lengths. Axons which had not been treated with DNP were found to contain a small amount of absorbing material which was equivalent to about 0·022 m-mole DNP/kg of axoplasm, and an allowance was made for it in working out the DNP contents.

*Substances used as chromatographic markers.* The ATP sample used was a sample of the potassium salt which had been obtained from the Sigma Chemical Company. Of the two arginine phosphate samples, one was a gift from Professor A. H. Ennor, and the other was a sample which had been prepared from *Palinurus vulgaris* muscle by methods based on those of Ennor, Morrison & Rosenberg (1956). The arginine and glycocyamine were commercial samples.

## RESULTS

### *The identity of the phosphate fractions present in squid axons*

The molybdate–$H_2S$ method of Hanes & Isherwood (1949) shows the presence of three main phosphate fractions on chromatograms of extracts of the axoplasm from unpoisoned squid axons. One of these fractions is orthophosphate, since it travels the distances expected for orthophosphate and reacts rapidly with the molybdate spray to form yellow phosphomolybdate before the chromatogram has been heated. (With chromatograms run in tert-butanol–picric acid the yellow spot formed by orthophosphate was obscured. If, however, the chromatogram was dried in a current of warm air after spraying with the molybdate reagent the spot became visible, since the other parts of the chromatogram went colourless.) The other two main fractions travel about the distances expected for ATP and arginine phosphate and have been assumed to consist of these compounds. The distances travelled by the ATP and arginine phosphate fractions in the solvents which were usually used for two-dimensional chromatography are given in Table 1, together with the distances travelled on two two-dimensional chromatograms by samples of authentic ATP and arginine phosphate. The distances given in the Table are in terms of the distance travelled by orthophosphate. The values given for the fractions in the axons are for extracts from unpoisoned axons, but closely similar distances were found when these fractions occurred in extracts of poisoned axons. It will be seen that the mean values for the distances travelled by the ATP and arginine phosphate fractions are close to the distances travelled by the authentic ATP and arginine phosphate.

TABLE 1. Distances travelled on two-dimensional chromatograms by the phosphate fractions in squid axoplasm and by ATP and arginine phosphate, as (Distance travelled)/(Distance travelled by orthophosphate)

| | Distance travelled | |
|---|---|---|
| | In *n*-propanol–ammonia | In tert-butanol–picric acid |
| Fractions in squid axoplasm: | | |
| ATP fraction | 0·81 | 0·13 |
| Arginine phosphate fraction | 1·10 | 0·65 |
| Unknown $\alpha$ | 0·52 | 0·10 |
| Unknown $\beta$ | 0·72 | 0·06 |
| Known substances: | | |
| ATP | 0·86 | 0·19 |
| Arginine phosphate | 1·19 | 0·71 |

Further tests were done to establish the identity of the ATP and arginine phosphate fractions. In the case of the ATP fraction the Gerlach & Döring (1955) test for adenine compounds was used on some chromatograms for

which tert-butanol–TCA had been used as the second solvent. The ATP spot gave the same positive reaction as authentic ATP, indicating that it consisted of an adenine-containing compound.

In the case of the arginine phosphate fraction, the following additional experiments were carried out. Chromatograms of extracts of axoplasm from unpoisoned axons were first run in *n*-propanol–ammonia in the normal way. Arginine phosphate, arginine and glycocyamine markers for the second dimension were then applied and the chromatograms were then heated at 100° C for 2 or 3 hr in order to decompose the arginine phosphate. The chromatograms were then run in the second dimension in solvents suitable for the separation of the guanidine bases found in invertebrate phosphagens. The bases were detected on the chromatograms by the method described by Hobson & Rees (1955) for the detection of arginine-like bases. In all three solvents used the base from the squid phosphagen had travelled the same distance as the arginine markers and the arginine liberated from the arginine phosphate markers. In one of the solvents, (*n*-butanol 63–formic acid 20–water 17; Roche, Thoai & Hatt, 1954) the glycocyamine spots were well separated from the arginine spots. Taurocyamine was unfortunately not available, but the other two solvents used (*n*-propanol–ammonia and *iso*pentanol 40–pyridine 80–water 70) are stated (Roche *et al.* 1954; Hobson & Rees, 1955) to give good separations of taurocyamine from arginine. It therefore seems almost certain that the phosphagen in squid giant axons is arginine phosphate and not glycocyamine phosphate or taurocyamine phosphate. That it is not creatine phosphate or lombricine phosphate is shown by the fact that the distances travelled by these substances relative to orthophosphate in the *n*-propanol–ammonia solvent (1·5 for creatine phosphate; 0·7 for lombricine phosphate; Caldwell, 1953; Rey, 1956) are appreciably different from the distance travelled by the phosphagen.

In addition to the three main phosphate fractions, two further spots corresponding to phosphate fractions, which have not yet been identified, were sometimes shown up by the Hanes & Isherwood molybdate reagent. These have been called Unknowns $\alpha$ and $\beta$. Unknown $\alpha$ was found more often than Unknown $\beta$ and it is possible that in some cases the two fractions did not separate. The distances travelled by Unknowns $\alpha$ and $\beta$ in *n*-propanol–ammonia and tert-butanol–picric acid are given in Table 1. A spot corresponding to ADP was not normally found, although in a few cases there was a faint indication of one. Phosphorus analyses of the region of the chromatograms corresponding to ADP, however, usually showed the presence of some phosphorus and this is included in Table 2 as the 'ADP' fraction.

*Amounts of phosphorus present in the phosphate fractions of squid axoplasm*

The results of phosphorus analyses obtained for chromatograms of extracts of axoplasm from unpoisoned axons are given in Table 2. In addition to the figures for orthophosphate, ATP, arginine phosphate, Unknown $\alpha$, Unknown $\beta$ and the non-extractable phosphorus, the Table includes the figures which were obtained for the regions of the chromatograms in which certain phosphate fractions might have been present, even though the Hanes & Isherwood molybdate reagent had not indicated their presence. Very little phosphorus was found in these latter regions and it is

TABLE 2. Distribution of phosphorus in squid axoplasm

| Phosphate fraction | No. of determinations | Mean amount present and range ($\mu$g P/g) |
|---|---|---|
| Orthophosphate | 16 | 117 (66–232) |
| Arginine phosphate | 16 | 105 (40–161) |
| ATP | 16 | 125 (100–191) |
| Unknown $\alpha$ | 14 | 26 (13–46) |
| Unknown $\beta$ | 1 | 14 |
| 'ADP' fraction | 8 | 21 (13–34) |
| Monophosphate esters | 7 | 7 (0–17) |
| Diphosphate esters | 6 | 4 (0–9) |
| Unknown B* | 6 | 6 (0–17) |
| Total phosphorus extracted by TCA | — | 425 |
| Phosphorus not extracted by TCA | 9 | 293 (238–335) |
| Total phosphorus accounted for | — | 718 |

* Unknown B refers to an unidentified fraction which was found in extracts of tortoise muscle (Caldwell, 1953).

doubtful in most cases whether any phosphate compounds were present. No conclusions could be drawn about possible changes in any compound in these regions or about any possible changes in Unknown $\beta$ when the axons were poisoned.

It will be seen from Table 2 that about 718 $\mu$g/g of the phosphorus in the axoplasm can be accounted for. Two analyses of the total phosphorus in the axoplasm, one for an axoplasm sample from an unpoisoned axon and the other for an axoplasm sample from an axon which had been soaked for about 95 min in sea water of pH about 8 containing 0·2 mM DNP gave values of 790 $\mu$gP/g and 700 $\mu$gP/g. Keynes & Lewis (1951) obtained a mean value of 838 $\mu$gP/g for the phosphorus in squid axoplasm by means of activation analysis. It seems therefore that nearly all the phosphorus in the axoplasm can be accounted for.

*The effects of* 2 mM *cyanide on the phosphate compounds in the axons*

In these experiments axons were immersed for varying periods in sea water containing 2 mM cyanide. In some cases they were transferred,

after 90–95 min soaking, to cyanide-free sea water. In some of the experiments the sea water circulating in the laboratory tanks (circulation sea water) was used, and in the other experiments an artificial sea water (bicarbonate sea water) with the following composition (mM): NaCl 460; $MgCl_2$ 55; $CaCl_2$ 11; KCl 10·4; $NaHCO_3$ 2·5. The pH of the samples of circulation sea water used in the experiments was not measured, but

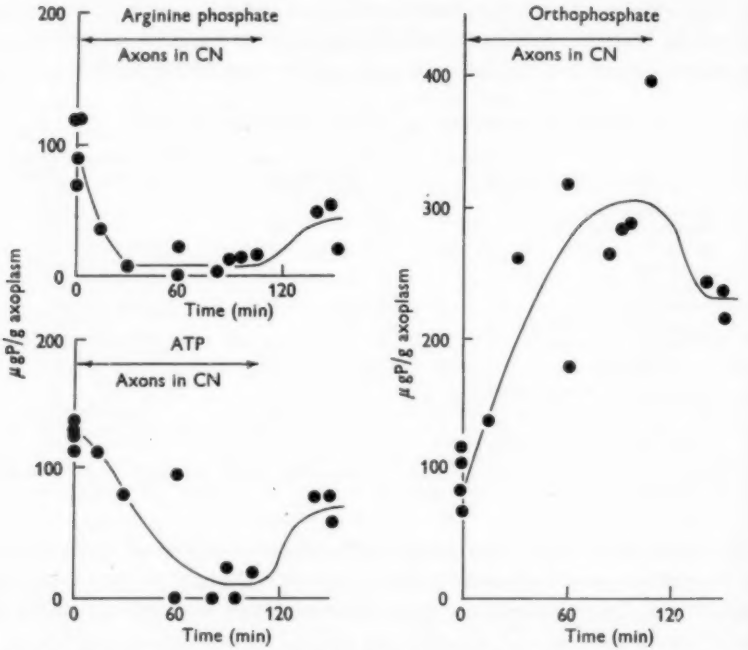


Fig. 1. The time course of the effects of 2 mM cyanide on the amounts of arginine phosphate, ATP and orthophosphate in squid axons. The results obtained with axons which were soaked only in sea water containing cyanide are indicated. The three axons which were soaked for a total of 140–152 min were soaked first for 90–95 min in sea water containing cyanide and were then transferred to cyanide-free sea water for a further period of soaking.

subsequent measurements have indicated that the pH of the cyanide-free circulation sea water was probably about 8·0 and that the pH of the circulation sea water containing cyanide (which was added as NaCN) was probably about 8·8. The pH of the bicarbonate sea water used in these experiments was 8·2–8·5 and the pH of the bicarbonate sea water containing cyanide was 7·5–8·5.

The results of the experiments are given in Fig. 1. The values for the

amounts of the various phosphate fractions present at zero time given in this figure and also those given in Figs. 2 and 3 are for axons which had been soaked for 15–135 min in inhibitor-free sea water. It will be seen that ATP and arginine phosphate disappear from the axons on immersion in cyanide and that these compounds reappear when the cyanide is removed. The time course for these changes is similar to that for the changes which

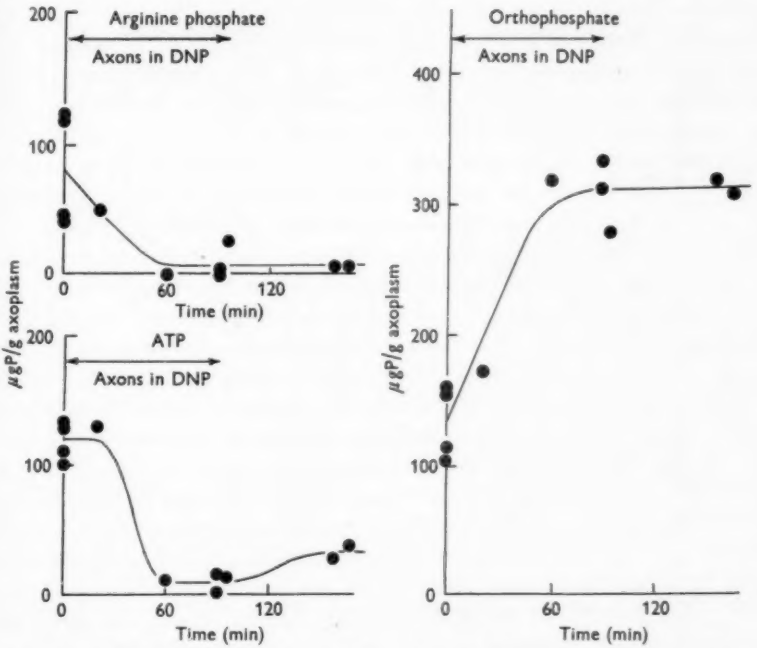


Fig. 2. The time course of the effects of 0·2 mM DNP at pH 6·5–6·7 on the amounts of arginine phosphate, ATP and orthophosphate in squid axons. The results obtained with axons which were soaked only in DNP-containing sea water are indicated. The two axons which were soaked for a total of 157–166 min were soaked first for 91 min in sea water containing DNP and were then transferred to DNP-free sea water for a further period of soaking.

cyanide brings about in the sodium efflux from squid axons (see, for example, Caldwell, Hodgkin, Keynes & Shaw, 1960*a*). In addition to the results given in Fig. 1 values were obtained for the Unknown α and the non-extractable phosphorus in the axons. No significant changes were detected in the non-extractable phosphorus. Indications were, however, obtained that the amount of Unknown α decreased when the axons were treated with cyanide.

*The effects of* 0·2 mM *DNP on the phosphate compounds in the axons*

Sea waters of two different pH ranges were used in these experiments and it was found that the effects of 0·2 mM DNP on the phosphate compounds in the axons varied with pH. In one series of experiments the artificial sea water used had the same composition as that used by Hodgkin & Keynes (1955). The pH of this sea water, which will be referred to as acid

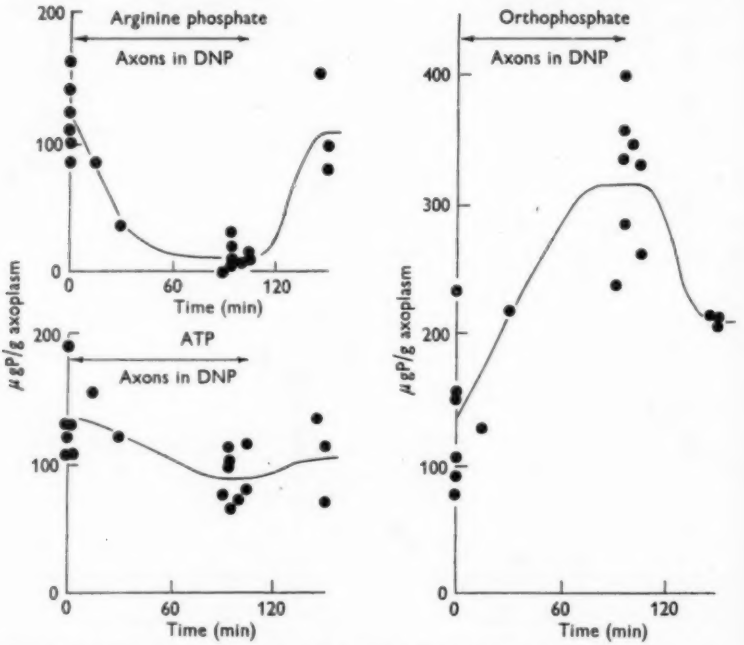


Fig. 3. The time course of the effects of 0·2 mM DNP at a pH of about 8 on the amounts of arginine phosphate, ATP and orthophosphate in squid axons. The results obtained with axons which were soaked only in DNP-containing sea water are indicated. The three axons which were soaked for a total of 145–150 min were soaked first for 95–100 min in sea water containing DNP and were then transferred to DNP-free sea water for a further period of soaking.

sea water, was found to be between 6 and 7, both before and after the addition of DNP which was added as the sodium salt. In the second series of experiments either circulation sea water or bicarbonate artificial sea water were used, the pH of these usually being near 8.

In Fig. 2 the effects of the immersion of axons in the acid sea water containing 0·2 mM DNP are shown, together with the changes which follow the reimmersion of the axons in DNP-free acid sea water. The pH

of the acid sea water used in the experiments on changes in the phosphate compounds was 6·5–6·7, both with and without DNP. It will be seen from Fig. 2 that the pattern of changes for axons treated with 0·2 mM DNP in the acid sea water is similar to that found with axons treated with cyanide in circulation sea water or bicarbonate sea water. The main difference is that there appears to be little resynthesis of the arginine phosphate when the DNP-treated axons are transferred to DNP-free acid sea water. As in the case of cyanide-poisoned axons, the time course for the changes in the phosphate esters is similar to that for the changes in the sodium efflux which is, under these pH conditions, like that in 2 mM cyanide (Caldwell, Hodgkin, Keynes & Shaw, 1960*b*). In these experiments no significant changes were found in the non-extractable phosphorus, but indications were obtained that Unknown $\alpha$ decreased when the axons were treated with 0·2 mM DNP in the acid sea water.

In Fig. 3 the effects of the immersion of the axons in circulation sea water or in bicarbonate sea water containing 0·2 mM DNP are shown. The pH of the bicarbonate sea water used was 7·9–8·1 and that of the bicarbonate sea water containing 0·2 mM DNP was 7·8–8·1. In most of the experiments, however, circulation sea water was used and the pH of the samples actually used in the experiments was not measured. The samples of circulation sea water containing 0·2 mM DNP were made up by a tenfold dilution with circulation sea water of a 2 mM solution of DNP in circulation sea water. The pH of a 2 mM solution of DNP in circulation sea water which had been used for this purpose was measured at a later stage. The pH of a tenfold dilution of this with circulation sea water of pH 8·0 was also measured. The pH values of both these solutions were found to lie between 7·8 and 8·1 and there seems little doubt that the pH of the circulation sea water and of the circulation sea water containing DNP used in the experiments shown in Fig. 3 must have been close to this range of values. It will be seen that under these pH conditions 0·2 mM DNP has only a small effect on the level of ATP in the axons but that the amount of arginine phosphate is markedly decreased. The main effect of 0·2 mM DNP on the sodium efflux under these pH conditions is to remove the potassium sensitivity of the sodium efflux without greatly reducing the level of the efflux when the usual amount of potassium is present (Caldwell *et al.* 1960*b*). It seems likely therefore that the disappearance of the arginine phosphate may be linked with this decrease in potassium sensitivity. Some values were obtained for the amount of Unknown $\alpha$ in the axons which had been immersed in the circulation sea water containing 0·2 mM DNP and these indicated that the level of Unknown $\alpha$ is not seriously affected.

An experiment was also done in which an axon was soaked for 90 min

in acid sea water (pH 6·7) containing 0·2 mM DNP and was then soaked for 77 min in circulation sea water (pH 8·4) containing 0·2 mM DNP. On analysis of the axoplasm 60 $\mu$g/g phosphorus was found in the ATP fraction and this presumably had been resynthesized after the axon had been transferred from the acid sea water containing DNP to the circulation sea water containing DNP. On the other hand, no arginine phosphate was found, a result which is consistent with those in Fig. 3.

*The effect of pH on the amount of DNP taken up by squid axons*

The axoplasm from axons which have been immersed in acid sea water containing 0·2 mM DNP is much more markedly yellow in colour than that from axons which have been immersed in circulation sea water containing 0·2 mM DNP. Some colorimetric determinations were carried out of the DNP content of axoplasm samples from axons which had been immersed in one or other of these DNP-containing sea waters for 80–107 min. In five determinations carried out on axoplasm from axons which had been soaked in DNP-containing sea waters of pH 6·5–6·8 (four determinations for axons in acid sea water, one for an axon in circulation sea water acidified with HCl) a mean DNP content of 0·29 m-mole DNP/kg (range 0·25–0·35 m-mole/kg) was found. In four determinations on axoplasm from axons which had been soaked in DNP-containing circulation sea water of pH 8·0–8·2 a mean DNP content of only 0·04 m-mole DNP/kg (range 0·03–0·07 m-mole/kg) was found. These results suggest therefore that the differences between the effects of 0·2 mM DNP on the phosphate compounds shown in Fig. 2 and those shown in Fig. 3 may be due to an effect of pH on the ease with which DNP penetrated into the axons. Much more DNP must have penetrated into the axons in the experiments shown in Fig. 2 than in the experiments shown in Fig. 3 and this resulted in more marked effects on the levels of the phosphate compounds.

As was pointed out by Krahl & Clowes (1938), the most reasonable explanation of the effects of pH on the penetration of DNP is that the axon membrane is impermeable to the negatively-charged dissociated form of DNP but is permeable to the undissociated form. The amount of the latter will, under the experimental conditions used, decrease with increasing pH and hence less DNP should penetrate into the axons as the external pH increases. It can easily be shown from a consideration of the dissociation constant of DNP that in the pH range used in the present experiments, $\log_{10}$ (DNP concentration in the axon)/(DNP concentration in the sea water) should be approximately equal to (pH in the axon − pH in the sea water) when the DNP has become equilibrated between the axon and the sea water. The amounts of DNP taken up under the different pH conditions indicate a range of 6·6–7·6 for the pH inside the axons and

this is consistent with the range of 6·7–7·35 obtained from glass electrode measurements (Caldwell, 1958*c*).

### *The effects of* 3 mM *azide and* $10^{-5}$ M *ouabain on the phosphate compounds in the axons*

The effects of 3 mM azide on the sodium efflux from squid giant axons do not appear to have been investigated, but Hodgkin & Keynes (1955) found that the $^{24}Na$ efflux from *Sepia* giant axons immersed in artificial sea water containing this concentration of azide was markedly lowered. The following figures ($\mu$gP/g axoplasm) were obtained for the phosphate compounds in two axons which had been immersed for 150 min in circulation water containing 3 mM sodium azide: ATP, 69 and 0; arginine phosphate, 20 and 0; orthophosphate, 327 and 293. A third axon was immersed for 150 min in circulation water containing 3 mM sodium azide and was then immersed for 55 min in azide-free circulation water. The figures ($\mu$gP/g) obtained for the phosphate compounds were : ATP, 58; arginine phosphate, 64; orthophosphate, 142. These results indicate that 3 mM azide brings about a break-down of the arginine phosphate and to a less extent of the ATP. They also indicate that some resynthesis takes place when the azide is removed. It seems likely that, as in the cases of cyanide and DNP, there is some relationship between the effect of azide on the levels of the 'high energy' phosphate compounds and its action on the sodium efflux.

Two axons were exposed to $10^{-5}$ ouabain. One was immersed for 125 min in bicarbonate sea water containing $10^{-5}$ M ouabain and the other was immersed for 158 min in circulation sea water containing $10^{-5}$ ouabain. The following figures ($\mu$gP/g) were obtained for the phosphate compounds: ATP, 134 and 114; arginine phosphate, 130 and 53; orthophosphate, 78 and 88. Although the sodium efflux of these two axons must have been greatly reduced by the ouabain (Caldwell & Keynes, 1959), there does not appear to have been any significant reduction in the levels of ATP and arginine phosphate and in this respect the action of ouabain on the axons differs from the effects of cyanide, DNP and azide. An attempt was also made with a pair of axons to see whether $10^{-5}$ M ouabain modifies the rate at which the 'high-energy' phosphate compounds break down in axons immersed in sea water containing 2 mM cyanide. The results of this experiment indicated however that ouabain does not have any marked effect on the rate of break-down.

## DISCUSSION

The work described in this paper indicates that in the case of squid axons poisoned with cyanide or DNP (at pH 6·5–6·7) there is a correlation between the presence in the axons of the 'high-energy' phosphate

compounds, ATP and arginine phosphate, and the ability of the axons to extrude sodium. Results which suggest that a similar correlation exists between the ATP content of red cells and their ability to bring about the active transport of potassium and sodium have been obtained by a number of workers (e.g. Prankerd, 1956; Fleckenstein, Gerlach & Janke, 1956; Whittam, 1958). The decrease of creatine phosphate and ATP produced by stimulating mammalian C fibres at 50/sec for 15 sec (Greengard & Straub, 1959) is also consistent with these compounds being involved in the extrusion of sodium. In the case of squid axons the correlation has been confirmed and extended in subsequent work (Caldwell *et al.* 1960*a*) in which the sodium efflux, and in some cases the potassium influx, of poisoned axons have been restored by injections of the 'high-energy' phosphate compounds ATP, ADP, arginine phosphate and phosphoenol-pyruvate. There seems to be little doubt therefore that the active transport of sodium from the axons is very closely linked with the metabolism of the 'high-energy' phosphates and it is possible that one or more of these may participate in the transport mechanism.

In contrast to cyanide, DNP and azide, which appear to act on sodium transport by interfering with the formation of 'high-energy' phosphate bonds, ouabain appears to act more directly on the actual transport mechanism. Thus $10^{-5}$M ouabain drastically reduced the sodium efflux from the axons in a matter of minutes (Caldwell & Keynes, 1959) whereas no significant changes in the ATP and arginine phosphate contents have been found after 125–158 min treatment with $10^{-5}$M ouabain. This result is analagous to that obtained by Whittam (1958) in work on the effects of digoxin and ouabain on the potassium accumulation and phosphate metabolism of red cells. That ouabain acts more directly on the sodium transport mechanism than cyanide and DNP is shown by the fact that it inhibits the increased sodium efflux which is normally observed when ATP is injected into axons poisoned with cyanide (Caldwell & Keynes, 1959). It is also of interest that ouabain appears to act only on a part of the transport mechanism taking place on the outside of the axons (Caldwell & Keynes, 1959), since this is in contrast to the effects of DNP which seem to depend on the amount of DNP penetrating into the axons.

Finally, the question of the break-down products formed by ATP when the axons are poisoned must be mentioned. On the two-dimensional chromatograms obtained with extracts from axons poisoned with cyanide or DNP at pH 6–7 there was no indication of the formation of ADP and no conclusive evidence was obtained for the formation of AMP from analyses of the area of the chromatograms in which AMP should have occurred. However, in more recent work with extracts from axons poisoned with cyanide (Caldwell, Hodgkin, Keynes & Shaw, unpublished) a spot

which is positive to the Gerlach & Döring (1955) adenine test and which travels the same distance as an AMP marker has been found on chromatograms which were run successively in one dimension in *n*-propanol–ammonia and tert-butanol–TCA. It seems likely therefore that some of the ATP in poisoned axons breaks down to AMP.

## SUMMARY

1. The phosphorus compounds in squid giant axons have been studied. The four main phosphorus fractions are ATP, arginine phosphate, orthophosphate and the phosphorus which is not extracted from the axon debris by 5% trichloroacetic acid.

2. When squid axons are immersed in sea water containing 2 mM cyanide there is a considerable fall in the amounts of ATP and arginine phosphate in the axoplasm. When the cyanide is washed away again there is some resynthesis of the ATP and arginine phosphate.

3. When the axons are immersed in sea water of pH 6·5–6·7 containing 0·2 mM DNP there is a considerable fall in the amounts of ATP and arginine phosphate in the axoplasm. When the DNP is washed away again there is some resynthesis of ATP but there does not appear to be resynthesis of arginine phosphate.

4. When the axons are immersed in sea water of pH about 8 containing 0·2 mM DNP there is a marked decline in the amount of arginine phosphate in the axoplasm but only a slight fall in the amount of ATP. The arginine phosphate is restored to its original level when the DNP is washed away.

5. Measurements of the DNP taken up by the axons have shown that much more DNP is taken up at pH 6·5–6·8 than at pH 8·0–8·2 and it seems that this may be the reason for the different effects of 0·2 mM DNP at the two pH values.

6. The changes in ATP and arginine phosphate brought about by cyanide and DNP appear to run parallel with the changes which these inhibitors bring about in the sodium efflux, and this suggests that ATP and arginine phosphate may play some part in the active transport of sodium.

7. Ouabain ($10^{-5}$ M in sea water) in contrast to cyanide and DNP has no significant effect on the ATP and arginine phosphate in the axons, even though at this concentration it brings about a marked inhibition of the sodium efflux. Azide (3 mM in sea water) acts in the same way as cyanide and DNP.

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# THE EFFECTS OF INJECTING 'ENERGY-RICH' PHOSPHATE COMPOUNDS ON THE ACTIVE TRANSPORT OF IONS IN THE GIANT AXONS OF *LOLIGO*

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In the previous paper (Caldwell, 1960) it was shown that the phosphagen and adenosine triphosphate (ATP) of squid giant axons break down in the presence of fairly high concentrations of inhibitors such as cyanide, dinitrophenol or azide and that some recovery occurs when the inhibitors are removed. Since these agents also reduce the outflow of sodium ions from giant axons to a low value (Hodgkin & Keynes, 1955) it is natural to suppose that ATP or some other energy-rich phosphate compound may provide the energy for running the ionic transport system. This would fit with the generally accepted view as to the function of ATP in linking biochemical and physiological events in cells (Lipmann, 1941). The aim of the experiments described here was to test the assumption by seeing whether injected ATP or arginine phosphate could restore normal ion transport to a fibre poisoned with cyanide or dinitrophenol. The results show that both arginine phosphate and ATP have a restorative action, but that the former is a more effective substitute for normal metabolic activity. One of the properties of the ionic transport system is that the outflow of sodium ions from a normal fibre is reduced to about one quarter by removing potassium ions from the external medium (Hodgkin & Keynes, 1955). This may happen because metabolism drives a cycle in which an uptake of $K^+$ is coupled to an extrusion of $Na^+$. Whatever the mechanism, it was plainly important to determine whether the sodium efflux which resulted from the injection of a phosphate ester had the same potassium sensitivity as the normal efflux. It turns out that high concentrations of certain compounds, arginine phosphate and phosphoenolpyruvate, restore K sensitivity, but that others, ATP and ADP, give an outflow of sodium which is not reduced by removing K.

The experiments described here were carried out during the last four months of the years 1956–59. Preliminary accounts by the authors

concerned with different phases of the work were given at various meetings and symposia (Caldwell & Keynes, 1957; Caldwell, 1958; Keynes, 1958; Caldwell, Hodgkin & Shaw, 1959).

## METHODS

*Material.* Giant axons from *Loligo forbesi* with diameters varying between 600 and 1000 $\mu$ were isolated and cleaned by the usual methods. Axons of over 700 $\mu$ are found in the large specimens of *L. forbesi* which are plentiful at Plymouth during the last 3 months of the year. These large squid are often damaged in the trawl or while being transported to the laboratory. A further hazard is rough weather which sometimes makes it difficult to bring any live squid to the laboratory. About half the work was done with live squid from the aquarium; these were normally used on the day that they were caught. The remaining experiments were

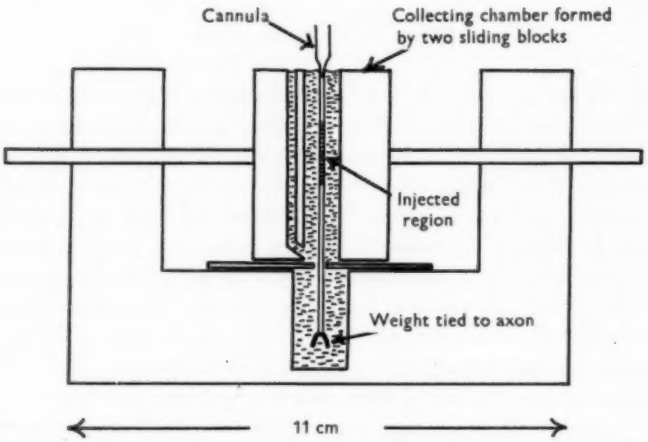


Fig. 1. Collecting cell for measuring the efflux of radioactive ions from squid axons. During the impalement of the axons with a micro-electrode or micro-syringe, the sliding blocks were pulled apart in order to allow more room for manoeuvre. They could be pushed together again with the microsyringe in position inside the axon, their sides being well greased with petroleum jelly so that they formed a watertight rectangular compartment whose volume (above the lower gate) was about 4 ml. Chlorided silver wires (not shown in the diagram) were used to apply shocks between the two compartments, thus stimulating the axon at the point where it passed through the gate. The drawing is roughly to scale, the over-all length of the cell being 11 cm, its height 7 cm, and its width 4 cm.

carried out with axons from refrigerated mantles. When the trawl was brought up, live squid were decapitated, and after removing the viscera the mantles were placed in large Thermos flasks filled with ice-cold sea water. Axons were dissected a few hours later, a bright light being used to illuminate the opaque mantle; they seemed to be in good condition since they gave spikes of 105–110 mV for many hours.

*Procedure for injections and for measuring effluxes.* The fibre was cannulated and mounted in the cell shown in Fig. 1. A long electrode consisting of a 100 $\mu$ capillary filled with isotonic KCl was inserted for a distance of 3–4 cm. At this stage the sliding blocks of Perspex were separated so as to allow the axon to be viewed from the front and side, a mirror being

employed in the usual way. The action potential was measured and the impalement was regarded as satisfactory if the spike amplitude was greater than 105 mV over a length of 2 cm; it was also desirable that the micro-electrode should lie fairly near the axis of the fibre. The micro-electrode was removed and a microsyringe of the type described by Hodgkin & Keynes (1956) was lowered into the fibre to a distance of about 35 mm from the tip of the cannula. The microsyringe had previously been filled with a solution containing $^{22}Na$ and this was injected uniformly over a distance of 10 or 12 mm. The microsyringe was then withdrawn, washed thoroughly with distilled water and reloaded with phosphate ester. The tip of the syringe was lowered to the end of the region injected with $^{22}Na$, or, in the later experiments, to a point 3 mm beyond this region. Diffusion of phosphate ester from the tip of the syringe was prevented by a small air bubble in the tip. The movable blocks which had previously been greased with petroleum jelly were then pressed together so that the axon was enclosed in a volume of 4 ml. Samples were collected by sucking the cell dry with a syringe; this removed about 97 % of the fluid. If the composition of the solution had to be changed the cell was washed with the new solution before taking a sample; as a control the same procedure was often used when there was no change in composition. After collecting $^{22}Na$ from the normal fibre, cyanide or DNP was applied. After 90 min the Na efflux had always fallen to a low value and the time had come to observe the effect of the injection. Since the microsyringe was already in position, all that had to be done was to inject the column of phosphate ester and to continue collecting samples in the same way as before. The exact procedure varied slightly during the course of the work. In 1956 and 1957 the columns of $^{22}Na$ and phosphate ester were both 12 mm in length. Since about 2·5 hr elapsed between the two injections, the patch of $^{22}Na$ must have spread longitudinally by diffusion and some of the labelled ions would not have been reached by the phosphate ester; calculations in which a diffusion coefficient of $10^{-5}$ cm$^2$/sec was used indicated that, after 2·5 hr, 28 % of the total $^{22}Na$ (p. 569) would have been outside the column of phosphate ester. In 1958 the column of $^{22}Na$ was reduced to 10 mm and the column of phosphate ester increased to 16 mm, giving an overlap of 3 mm at either end. Under these conditions 12 % should have diffused beyond the column of phosphate ester in 2·5 hr. The corresponding figure for the 1959 experiments, in which the column of $^{22}Na$ was 12 mm and of ~ P was 18 mm, is 10 % in 2·5 hr.

The action potential was checked from time to time and the experiment was discontinued if there was any sign of block or decrement in the injected region. This was important because control injections of salt solutions which have no effect on excitable axons may give an apparent rise in efflux in derelict axons. (These spurious effects may sometimes be due to fluid being squeezed out of the ends of branches.)

At the end of the experiment the fibre was removed and the total quantity of $^{22}Na$ was measured. In many experiments the concentrations of Na and K in extruded axoplasm were also determined by flame photometry.

*External solutions.* The artificial sea water normally used was the bicarbonate sea water described in the previous paper (Caldwell, 1960, p. 552); its pH was about 8. In some experiments with 0·2 mM DNP a phosphate sea water of a lower pH (*ca.* 6·7) was made up with the composition given by Hodgkin & Keynes (1955).

*Phosphate compounds used for injection.* The solutions used for injection were adjusted to a pH of about 7 with KOH or HCl and were stored at $-20°$ C. At the end of each group of experiments the solutions were tested chromatographically for impurities, the amounts of phosphorus present as the principal component and as the main impurities being determined by the method described previously (Caldwell, 1960). Table 1 gives information about the source and purity of the various solutions used; the figures for the impurities refer to the final composition of the solutions and not to the products supplied by the manufacturers.

*Radioactive isotopes.* $^{22}Na$ was obtained from the Radiochemical Centre, Amersham, as an aqueous solution of $^{22}NaCl$. After concentration by evaporation, the pH was adjusted

to 7, and any precipitate was removed. The amount of inactive Na in the sample varied from year to year; the calculated increase in the internal sodium concentration produced by the injection was usually between 5 and 30 mM.

Artificial sea water containing $^{24}Na$ or $^{42}K$ was made by treating $^{24}Na_2CO_3$, $^{42}K_2CO_3$ or $^{42}KHCO_3$, supplied by AERE Harwell, with a slight excess of HCl, heating to dryness and adding a solution containing the other constituents of artificial sea water.

Except in the 1959 experiments, in which $\gamma$ rays from $^{22}Na$ were detected with a Labgear scintillation counter, the methods of counting liquid samples or the axon were essentially similar to those used by Hodgkin & Keynes (1955).

TABLE 1

| Substance | Year used | Source | K or Na salt | Main impurities (as a % of the P in the principal constituent) |
|---|---|---|---|---|
| Arginine phosphate | 1956*A* | *J* | K | 2–8% as orthophosphate ($P_i$) |
| | 1956*B** | *P* | K | 0–6% as $P_i$ |
| | 1957* | *P* | K | Nil |
| | 1958* | *P* | K | Nil |
| ATP | 1956*A*† | *P* | K | 28% as ADP, < 2% as $P_i$ |
| | 1956*B*† | *P* | K | < 18% as ADP, < 9% as $P_i$ |
| | 1957*A*† | *P* | K | 18% as ADP, 9% as $P_i$ |
| | 1957*B* | *S* | K | 12% as ADP, 4% as $P_i$ |
| | 1958 | *S* | K | 33% as ADP, 23% as $P_i$ |
| | 1959 | *S* | Na, K | 4% as ADP |
| ADP | 1957‡ | *S* | K | 10% as ATP, 15% as $P_i$ |
| | 1958‡ | *S* | K | 33% as ATP, 15% as $P_i$ |
| | 1959 | *S* | Na, K | < 5% as ATP |
| Phosphoenol-pyruvate | 1958 | *S* | Na | No $P_i$ visible on chromatogram |
| | 1959 | *S* | Na | 6% as $P_i$ |
| Creatine phosphate | 1958 | *S* | Na, K | No $P_i$ visible on chromatogram |
| GTP | 1959 | *S* | Na, K | 4% as $P_i$, some GDP? |
| ITP | 1959 | *S* | Na, K | 6% as $P_i$, some IDP? |

Notes: * Made by a method based on that of Ennor, Morrison & Rosenberg (1956). † Made from the Ba salt obtained as a by-product in the arginine phosphate preparation. This was dissolved in dil. $HNO_3$, the ATP precipitated as the Hg salt, brought into solution again with $H_2S$ and reprecipitated as the oxine salt with acetone. The oxine salt was redissolved, the pH adjusted to 7 with KOH and after removal of the oxine with chloroform the ATP was precipitated as the K salt with acetone. ‡ Prepared from Ba salt supplied by Sigma Chemical Company. *J*, *Jasus* muscle, gift from Professor Ennor; *P*, *Palinurus* muscle; *S*, Sigma Chemical Co.; GTP, guanosine triphosphate; ITP, inosine triphosphate.

## RESULTS

### *The effect of injections on Na efflux in poisoned fibres*

*Active and inactive substances.* If a giant axon is immersed for 1½–2 hr in 2 mM-CN (pH 8) the arginine phosphate and ATP disappear from the axoplasm and the Na efflux is reduced to ¼–1/10 of its normal value. Under these conditions the Na efflux can be increased for a period of 30 min, or longer, by injecting arginine phosphate or ATP; similar solutions which had been hydrolysed by boiling to adenosine monophosphate (AMP) and inorganic phosphate, or to arginine and inorganic phosphate, were without effect when injected into poisoned fibres (Figs. 2 and 3). Control injections

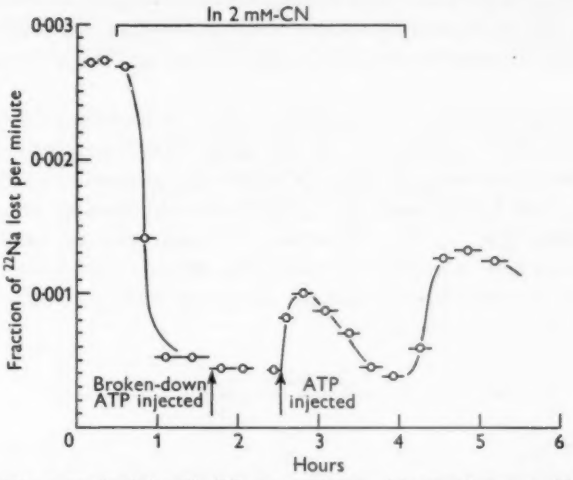


Fig. 2. 780 $\mu$ axon loaded with $^{22}$Na over 12 mm. At second arrow, 16·4 nmole ATP was injected over the same 12 mm, giving a mean internal concentration of 2·9 mM (5·8 mM ~ P). At the first arrow an equal volume of the same ATP solution which had been hydrolysed by boiling was injected. At the end of the experiment the action potential was 99 mV, and the resting potential 56 mV. Fibre reference, 19D6 (19 December 1956). 1 nmole = $10^{-9}$ mole.

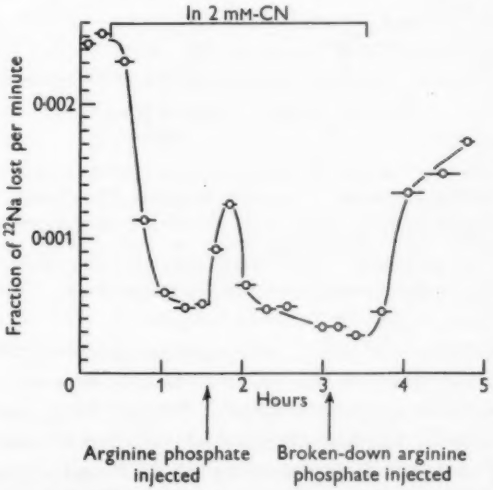


Fig. 3. 800 $\mu$ axon loaded with $^{22}$Na over 12 mm. At first arrow 32·4 nmole arginine phosphate was injected over same 12 mm, making mean internal concentration 5·4 mM. At second arrow an equal volume of the same solution, hydrolysed by heating, was injected. Temperature 20° C. At end of experiment resting potential was 58 mV, action potential 99 mV. Fibre reference, 4O6.

of KCl, $K_2SO_4$, K isethionate or $MgCl_2$ also gave negative results. Arginine phosphate and ATP had the same restorative effect on fibres poisoned with 0·2 mM dinitrophenol (DNP) (pH 6·7) as on those poisoned with 2 mM-CN.

In all, seven compounds containing energy-rich phosphate bonds have been tested, namely, arginine phosphate, ATP, ADP, phosphoenolpyruvate, GTP, ITP and creatine phosphate. The first five gave substantial increases in Na efflux but ITP caused only a slight rise and creatine phosphate was without effect (Fig. 4). The difference between creatine phosphate and arginine phosphate is to be expected if the effects of the latter depend on catalysis by arginine phosphokinase (Ennor & Morrison, 1958) or by an enzyme of similar specificity.

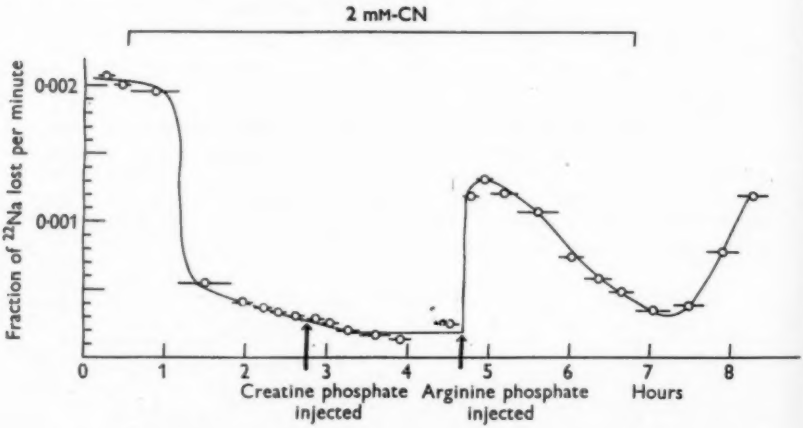


Fig. 4. The effect on the Na efflux from a CN-poisoned axon of injecting first creatine phosphate, and secondly arginine phosphate. The length injected with $^{22}$Na was 10 mm, and with phosphate ester 16 mm. The mean concentrations in the axon immediately after the injections were 15·3 mM creatine phosphate and 15·8 mM arginine phosphate. Axon 10D8, diameter 766 $\mu$. Temperature 19° C. At the end of the experiment the action potential was 90 mV.

*External applications of ATP and arginine phosphate.* Figure 5 shows that ATP had no effect on the sodium efflux of a poisoned fibre when applied externally at a concentration of 3·8 mM. Arginine phosphate at a concentration of 12 mM was also ineffective when applied externally.

*The effect of increasing concentrations of ATP and arginine phosphate.* The action of injected phosphate esters is transient, as one would expect if they are used up in driving ions against concentration differences, or destroyed in side-reactions. With quantities of arginine phosphate or ATP equivalent to about half the total energy-rich phosphorus in an

unpoisoned fibre the period of enhanced Na efflux lasted for about 30 min. With ten times the quantity, the Na efflux remained at a high level for several hours (e.g. Fig. 10).

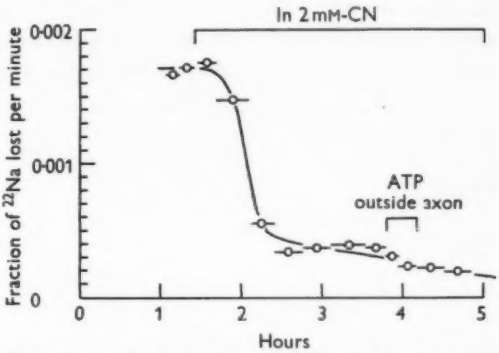


Fig. 5. The effect on the Na efflux from a CN-treated axon of applying ATP externally. This experiment was done with the collecting cell modified so that the external volume was 0·5 ml. During two collecting periods the CN sea water contained 3·8 mM ATP (7·6 mM ~ P). Axon 15N7, diameter 845 $\mu$. Temperature 18° C. Final resting potential was 52 mV, action potential 103 mV.

Figure 6 illustrates the effect of injecting first a small and secondly a larger quantity of arginine phosphate into the same fibre. In the first case the quantity of arginine phosphate injected was sufficient to give a concentration in the axoplasm of 4·4 mM (as compared with 1·5–5 mM in unpoisoned fibres); in the second, the concentration was 3·5 times larger. The experiment shows that the extra Na efflux resulting from the second injection reached a higher peak and lasted longer than that from the first. A similar experiment with two different concentrations of ATP is given in Fig. 7. In both cases (Figs. 6 and 7) the percentages of labelled Na which emerged as a result of the injection were roughly proportional to the quantity of energy-rich phosphate injected (Table 2).

One way of expressing the quantitative effect of injecting a substance is to give the ratio of the number of internal sodium ions ejected to the number of energy-rich phosphate bonds injected. In order to calculate such an Na: ~ P ratio it is necessary to estimate the internal sodium concentration at the time of the injection. Column 7 in Table 2 gives a series of such estimates. The values marked with an asterisk were obtained by interpolating between an initial sodium concentration taken as about 55 mM and the final sodium concentration measured by flame photometry on extruded axoplasm at the end of the experiment. The exact value taken for the initial sodium concentration depended on the time from decapitation and was based on analyses of unpoisoned axons made at

various times after death. The average of the values marked with an asterisk is 101 mM and an internal sodium concentration of 100 mM has been assumed in other cases. As a check, three pairs of axons were dissected and one axon of each pair was used for an injection experiment (31O8, 12N8 and 17N8 in Table 2); both were put into cyanide at the same time and the *control* was removed for analysis soon after its companion had

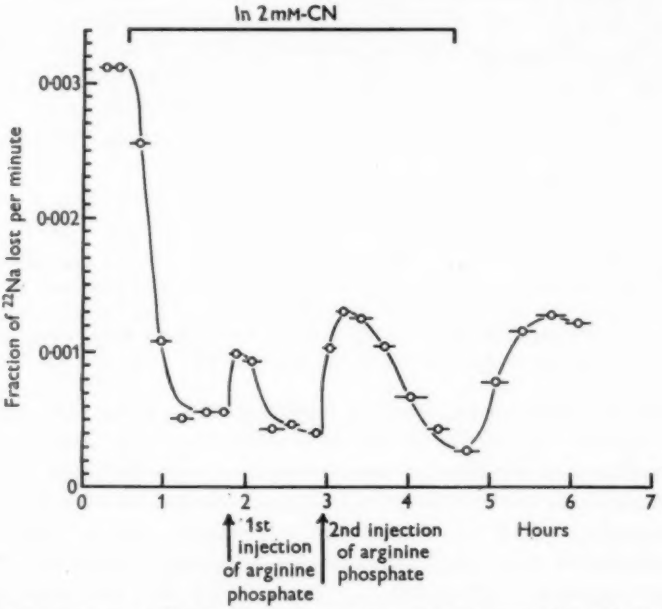


Fig. 6. The effect on Na efflux of injecting two different quantities of arginine phosphate. The axon was loaded with $^{22}$Na over a distance of 12 mm, and at the first arrow sufficient arginine phosphate to give an internal concentration of 4·4 mM was injected over the same 12 mm. At the second arrow a larger amount of arginine phosphate was injected, giving a concentration of 15·4 mM. Axon 5O6, diameter 668 $\mu$, temperature 17·5° C. At end of experiment resting potential was 59 mV, action potential 98 mV.

been injected. These axons had sodium concentrations of 94, 84 and 71 mM; to this should be added the quantity of Na injected with the $^{22}$Na. In these experiments the Na injected was of the order of 25 mM so that the estimate of 100 mM would seem to be roughly right.

From the estimates of $(Na)_i$, the fibre diameter and the percentage of internal sodium leaving the fibre as a result of the injection, it is simple to calculate the quantity of Na ejected and hence to obtain Na: ~ P. The values which are given in Column 9 of Table 2 are probably too small, because some $^{22}$Na would have diffused beyond the column ~ P. The

fraction ($F$) of the $^{22}Na$ which should have been outside the ~ P column can be calculated by the methods of Carslaw & Jaeger (1947) as

$$F = \frac{\sqrt{Dt}}{h}\left(\text{ierfc } l/2\sqrt{Dt} - \text{ierfc } \frac{2h+l}{2\sqrt{Dt}}\right), \quad (1)$$

Where $D$ is the diffusion coefficient of Na in axoplasm, taken, by analogy

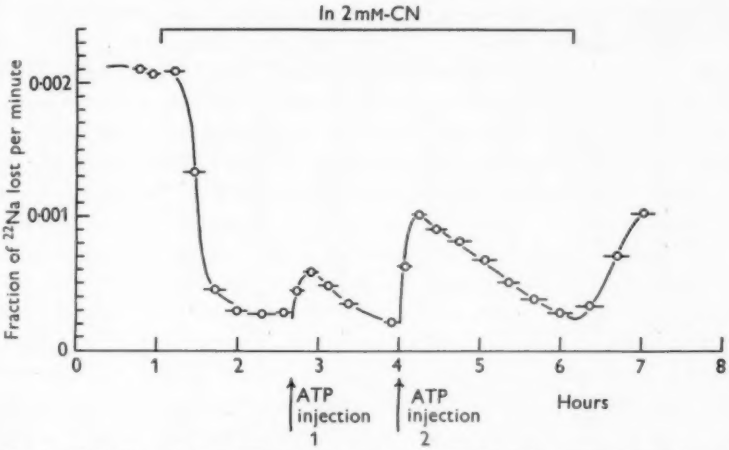


Fig. 7. Injection of two different amounts of ATP into the same axon. Axon loaded with $^{22}Na$ over 12 mm. Injection 1 was 5·9 nmole ATP over same 12 mm, making mean internal (ATP) 1·2 mM. Injection 2 was 31 nmole ATP, making mean internal (ATP) 6·2 mM. Axon 18D6, diameter 725 $\mu$. Temperature 19·5° C. At end of experiment resting potential was 47 mV, action potential 71 mV.

with K which has nearly the same mobility in axoplasm as in aqueous solution (Hodgkin & Keynes, 1953), as $10^{-5}$ cm$^2$/sec;

$2h$ is the total length of the column of $^{22}Na$;

$2h+2l$ is the total length of the column of ~ P;

$t$ is the time between the two injections; and

ierfc has its usual meaning (for definition and tables see Carslaw & Jaeger (1947), pp. 371–373). With the range of values involved, the second term in eqn. (1) is very small and can be neglected.

The corrected results, which are listed in Column 10 of Table 2, give mean values (± S.E. of mean) of Na: ~ P of 0·73 ± 0·06 for ATP, 0·40 ± 0·05 for ADP and 0·64 ± 0·06 for arginine phosphate. These values correspond to 1·46 Na per molecule of ATP, 0·40 Na per molecule of ADP and 0·64 Na per molecule of arginine phosphate.

The Na: ~ P ratios obtained for the other compounds were 0·7 and 0·8 for phosphoenolpyruvate, 0·27 and 0·47 for GTP and 0·03 for ITP.

There was no detectable ATP in the sample of ITP, but it is difficult to be sure that the slight effect obtained with ITP was not due to the presence of some other nucleotide.

TABLE 2. Relation between quantity ~ P injected and quantity Na extruded

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|
| Fibre reference | Temperature (°C) | Axon diameter (μ) | Initial concentration (mM ~ P) | Quantity ~ P injected (nmole/cm) | Na emerging (%) | Estimated $[Na]_i$ (mM) | Quantity Na ejected (nmole/cm) | Na: ~ P Uncorrected | Na: ~ P Corrected | |
| **ATP** | | | | | | | | | | |
| 18 D 6 | 20 | 725 | 2·4 | 9·8 | 1·1 | 100 | 4·6 | 0·47 | 0·63 | |
| 18 D 6 | 20 | 725 | 12·4 | 51 | 4·8 | 100 | 19·8 | 0·39 | 0·58 | |
| 20 N 7 | 19 | 890 | 2·8 | 17 | 1·5 | 103* | 9·6 | 0·56 | 0·79 | |
| 19 D 6 | 19 | 780 | 5·8 | 27 | 2·7 | 100 | 12·9 | 0·47 | 0·66 | |
| 19 D 7 | 18 | 911 | 7·4 | 49 | 4·2 | 98* | 27 | 0·55 | 0·78 | |
| 22 N 7 | 19 | 867 | 8·2 | 49 | 5·9 | 98* | 34 | 0·70 | 1·02 | 1 |
| 24 O 7 | 18 | 890 | 8·2 | 51 | 5·6 | 92* | 32 | 0·62 | 0·83 | 2 |
| 27 S 7 | 18 | 850 | 9·0 | 51 | 4·2 | 100* | 24 | 0·47 | 0·66 | 3 |
| 30 O 7 | 18 | 842 | 9·2 | 51 | 4·1 | 119* | 27 | 0·53 | 0·75 | 4 |
| 10 N 9 | 19 | 776 | 9·5 | 45 | 6·1 | 100 | 29 | 0·65 | 0·74 | |
| 4 O 7 | 17 | 830 | 9·6 | 51 | 6·5 | 124* | 44 | 0·85 | 1·30 | 5 |
| 11 D 6 | 18 | 581 | 12·6 | 33·6 | 4·3 | 100 | 11·4 | 0·34 | 0·46 | |
| 7 N 8 | 17 | 805 | 14·3 | 73 | 9·3 | 100 | 44 | 0·61 | 0·73 | |
| 12 S 7 | 19 | 578 | 19·6 | 51 | 4·9 | 102* | 13·1 | 0·26 | 0·34 | |
| Mean ± S.E. | | | 9·4 | | | | | | 0·73 ± 0·06 | |
| **ADP** | | | | | | | | | | |
| 11 N 9 | 17 | 806 | 2·1 | 11·5 | 0·6 | 100 | 3·0 | 0·26 | 0·30 | |
| 22 O 7 | 17 | 826 | 16·8 | 90 | 4·7 | 89* | 22·4 | 0·25 | 0·34 | |
| 7 N 7 | 18 | 796 | 18·0 | 90 | 6·1 | 85* | 25·8 | 0·29 | 0·42 | |
| 16 D 8 | 17 | 606 | 23·1 | 67 | > 9·6 | 100 | > 28 | *ca.* 0·45 | *ca.* 0·55 | |
| Mean ± S.E. | | | 15 | | | | | | 0·40 ± 0·05 | |
| **Arginine phosphate** | | | | | | | | | | |
| 5 O 6 | 18 | 668 | 4·4 | 15·3 | 1·1 | 100 | 3·8 | 0·25 | 0·33 | |
| 5 O 6 | 18 | 668 | 14·7 | 51·5 | 4·6 | 100 | 12·5 | 0·24 | 0·35 | |
| 4 O 6 | 20 | 800 | 5·4 | 27·0 | 1·5 | 100 | 7·5 | 0·28 | 0·37 | |
| 7 O 8 | 20 | 898 | 7·4 | 47 | 4·3 | 100 | 27 | 0·57 | 0·70 | |
| 21 N 8 | 16 | 844 | 8·5 | 47 | 2·0 | 100 | 11·3 | 0·24 | 0·28 | |
| 26 S 6 | 20 | 706 | 9·1 | 35·5 | 2·9 | 100 | 11·3 | 0·32 | 0·45 | |
| 20 S 6 | 21 | 664 | 10·3 | 35·5 | 3·4 | 100 | 11·7 | 0·33 | 0·43 | |
| 17 N 8 | 17 | 725 | 11·5 | 47 | 9·5 | 100 | 39 | 0·82 | 1·0 | |
| 13 D 7 | 18 | 690 | 12·1 | 45 | 10·6 | 100 | 39·6 | 0·88 | 1·23 | 2 |
| 24 S 6 | 22 | 578 | 13·5 | 35·5 | 5·0 | 100 | 13·1 | 0·37 | 0·51 | |
| 10 D 8 | 19 | 766 | 15·8 | 71 | 9·5 | 100 | 44 | 0·61 | 0·80 | |
| 31 O 8 | 17 | 810 | 18 | 90 | 9·8 | 100 | 50 | 0·56 | 0·83 | 6 |
| 28 O 8 | 19 | 840 | 22 | 122 | 12·5 | 100 | 68 | 0·56 | 0·63 | |
| 27 O 8 | 19 | 814 | 23·5 | 122 | 13·7 | 100 | 72 | 0·59 | 0·67 | |
| 5 N 8 | 17 | 834 | 24 | 133 | 16·8 | 100 | 92 | 0·69 | 0·75 | 6 |
| 21 O 8 | 17 | 761 | 27 | 122 | 12·7 | 100 | 58 | 0·47 | 0·57 | |
| 23 O 8 | 18 | 686 | 33 | 122 | 17·4 | 100 | 64 | 0·52 | 0·60 | |
| 12 N 8 | 16 | 800 | 37 | 188 | 31·8 | 100 | 160 | 0·85 | 1·02 | |
| Mean ± S.E. | | | 17 | | | | | | 0·64 ± 0·06 | |

Columns 6 and 8 give the quantities of Na emerging as a result of the injection; the residual efflux in cyanide has be subtracted. Na: ~ P ratios in column 10 have been corrected for diffusion of $^{22}Na$ beyond the phosphate column using a diffusion coefficient of $10^{-5}$ cm².sec. Concentrations (mM) are in m-mole/l. axoplasm. Since ATP contains 2 ~ per molecule the concentration in mM ~ P is twice the concentration in mM; for ADP and arginine phosphate, whi contain 1 ~ P per molecule, the two units are identical. Unless otherwise stated, 2 mM-CN and 10 mM-K were us 1 nmole=$10^{-9}$ mole.

Notes: * Estimated from analysis of axoplasm at the end of the experiment; in other cases a value of 100 mM has be assumed. 1. Preceded by injection of 0·3 M-$MgCl_2$. 2. 0·2 mM DNP at pH 6·8, 10 mM-K. 3. Preceded by injection 0·5 M-$MgCl_2$. 4. 0·2 mM DNP at pH 6·8, K-free. 5. 2 mM-CN, K-free. 6. 2 mM-CN, 20 mM-K.

Since the estimates of Na: ~ P are based on measurements of sodium efflux, rather than on net movements of sodium, they cannot be used to calculate the efficiency with which phosphate bond energy is transformed into secretory work. For example, if ATP increases Na influx as well as Na efflux, a calculation of efficiency based on efflux measurements would be erroneously large. An error in the opposite direction would occur if ATP and arginine phosphate are broken down in side reactions which have nothing to do with ion transport. In spite of these complications it is still interesting to see whether the observed Na: ~ P ratio can be checked by other methods. One method is to assume that cyanide blocks production of ATP rather promptly and that the lag in the fall of Na efflux depends on the energy-rich phosphate bonds stored in arginine phosphate and ATP. The fraction of internal sodium which leaves the fibre under the influence of the natural store of ~ P can be worked out from the area under the curve for the decline in the Na rate constant after applying cyanide; in measuring the area the basal efflux should, as usual, be subtracted from the efflux at any moment. The conclusion from analysing 29 experiments in Table 2 is that $5{\cdot}6 \pm 0{\cdot}3\,\%$ (mean and S.E.) of the labelled sodium moved out of the fibre during the initial period in cyanide. With an internal sodium concentration of 60–80 mM this is equivalent to a decrease in sodium concentration of 3·4–4·5 mM. From Caldwell (1960), the concentration of arginine phosphate is about 3·3 mM and of ATP about 1·3 mM (2·6 mM ~ P) so that Na: ~ P = 0·6–0·8. A similar figure is obtained by taking the approximate figure of 4Na per $O_2$, calculated (Hodgkin & Keynes, 1954) for *Sepia* axons and frog muscle; with a ~ P:$O_2$ ratio of 3 this gives Na: ~ P = 2:3. Since the three estimates all give roughly the same Na: ~ P ratio the tentative conclusion is that injected phosphate compounds may be used in the same way as those supplied by the normal metabolic cycle.

The Na: ~ P ratios described here are smaller than those calculated from the oxygen consumption and sodium transport of short-circuited frog skin (Zerahn 1956; Leaf & Renshaw, 1957). However, in the frog skin experiments, which gave Na: ~ P ratios of 3 if the basal oxygen consumption was subtracted, sodium ions were probably being transported against a lower electrochemical potential difference than that in nerve or muscle.

### *The extent to which the Na efflux is restored by injections*

*Arginine phosphate.* The relation between the initial concentration of arginine phosphate in the axoplasm and the absolute magnitude of the sodium efflux is illustrated by Fig. 8, curve *A*. In order to reduce scatter, the observations have been collected in groups according to the concentration

of ~ P. The efflux reached a saturating value of about 50 pmole/cm$^2$.sec at concentrations of 7–15 mM ~ P, and then remained constant. The values plotted were not corrected for longitudinal diffusion of $^{22}$Na,

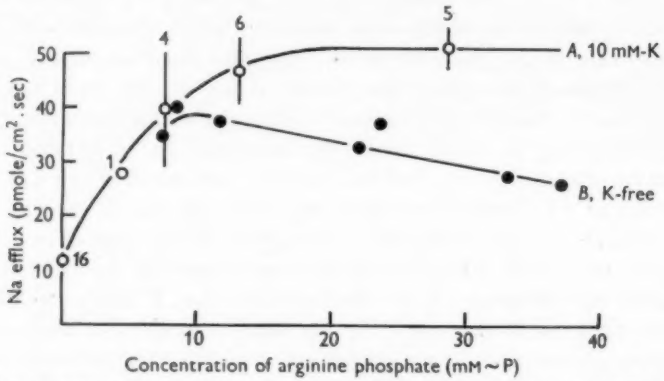


Fig. 8. Relation between initial concentration of injected arginine phosphate and peak Na efflux, ○, from observations in 10 mM-K; ●, from observations in K-free solution. The results of individual experiments in 10 mM-K have been collected in the following groups: < 5, 5–10, 10–20, 20–40 mM ~ P. ○ are the average of each group, the numbers in each group are shown against the points and ± 2 × S.E. of mean by the vertical lines. Values in K-free solutions (●) are individual results normalized by multiplying the ordinate of curve *A* by the figure for K sensitivity (Column 5 in Table 5).

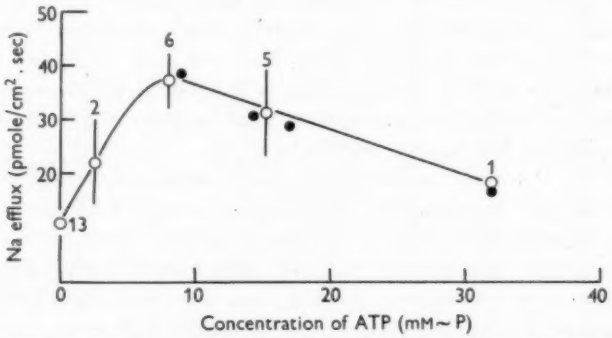


Fig. 9. Relation between initial concentration of ATP (mM ~ P) and peak sodium efflux. ○, from observations in 10 mM-K, averages after grouping as in Fig. 8; ●, from observations in K-free solution, obtained in the same way as in Fig. 8.

the reason being that since the efflux reaches saturation, longitudinal diffusion of arginine phosphate, which was neglected in deriving eqn. (1), would reduce the correction factor to an indeterminate extent. In con-

sidering the extent of restoration we wished to avoid any procedure which might exaggerate the effect of the injection. Further evidence of saturation was provided by the observation that with large doses of arginine phosphate the efflux often remained approximately constant for 1–3 hr and then declined relatively quickly (Fig. 10).

In order to decide whether arginine phosphate is fully restoring the sodium efflux, the best criterion is the rate constant for the loss of internal

TABLE 3. Rate constants and peak sodium fluxes after injection, arginine phosphate and phosphoenolpyruvate

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Rate constant ($min^{-1}$/1000) | | | | Extent of restoration | | | |
| Fibre reference | Temperature (°C) | Axon diameter ($\mu$) | Initial concentration (mM ~ P) | Normal, $k_1$ | Poisoned, $k_2$ | Injected, $k_3$ | Normal, $k_4$ | $\frac{k_3}{k_1}$ | $\frac{2k_3}{k_1 + k_4}$ | Peak Na efflux after injection (pmole/cm².sec) | Calculated correction factor |
| **Arginine phosphate** | | | | | | | | | | | |
| 5O6 | 18 | 668 | 4·4 | 3·1 | 0·50 | 0·99 | | 0·32 | 0·45 | 28 | 1·32 |
| 5O6 | 18 | 668 | 14·7 | | 0·35 | 1·28 | 1·3 | 0·41 | 0·58 | 36 | 1·45 |
| 4O6 | 20 | 800 | 5·4 | 2·5 | 0·48 | 1·26 | 1·7 | 0·52 | 0·60 | 42 | 1·32 |
| 7O8 | 20 | 898 | 7·4 | 1·65 | 0·26 | 1·40 | 1·76 | 0·85 | 0·82 | 52 | 1·24 |
| 21N8 | 16 | 844 | 8·5 | 1·14 | 0·29 | 0·74 | — | 0·65 | — | 26 | 1·17 |
| 28S6 | 20 | 706 | 9·1 | 3·5 | 0·32 | 1·40 | 1·5 | 0·40 | 0·56 | 41 | 1·41 |
| 20S6 | 21 | 664 | 10·3 | 3·6 | 0·61 | 1·93 | 2·1 | 0·54 | 0·68 | 53 | 1·30 |
| 17N8 | 17 | 725 | 11·5 | 1·65 | 0·36 | 1·42 | — | 0·86 | — | 43 | 1·22 |
| 13D7 | 18 | 690 | 12·1 | 3·5 | 0·53 | 1·98 | — | 0·57 | — | 57 | 1·41 |
| 24S6 | 22 | 578 | 13·5 | 3·35 | 0·58 | 2·06 | 2·7 | 0·62 | 0·68 | 50 | 1·39 |
| 10D8 | 19 | 766 | 15·8 | 2·0 | 0·18 | 1·32 | 1·2 | 0·66 | — | 42 | 1·31 |
| 31O8 | 17 | 810 | 18 | 2·9* | 0·44* | 1·92* | 1·2* | 0·66* | 0·94* | 65* | 1·48 |
| 28O8 | 19 | 840 | 22 | 2·1 | 0·35 | 1·62 | 1·5 | 0·77 | 0·90 | 57 | 1·12 |
| 27O8 | 19 | 814 | 23·5 | 2·1 | 0·38 | 1·54 | 1·45 | 0·73 | 0·87 | 52 | 1·14 |
| 1N8 | 17 | 834 | 24 | 3·0* | 0·35* | 1·94 | 1·5* | 0·65 | 0·86 | 67* | 1·09 |
| 21O8 | 17 | 761 | 27 | 2·1 | 0·30 | 1·60 | 1·4 | 0·76 | 0·91 | 51 | 1·22 |
| 22O8 | 18 | 686 | 33 | 2·2 | 0·37 | 1·85 | 1·6 | 0·84 | 0·97 | 53 | 1·15 |
| 12N8 | 16 | 800 | 37 | 1·3 | 0·35 | 1·35 | 1·1 | 1·05 | 1·12 | 45 | 1·20 |
| **Phosphoenolpyruvate** | | | | | | | | | | | |
| 24N9 | 16 | 804 | 17 | 1·52 | 0·23 | 1·00 | . | 0·66 | — | 47 | 1·14 |
| 28N8 | 15 | 860 | 20 | 1·51 | 0·27 | 1·14 | — | 0·76 | — | 57 | 1·22 |
| 2N8 | 16 | 844 | 21 | 1·14 | 0·34 | 0·83 | — | 0·73 | — | 41 | 1·36 |

In 20 mM-K, 13D7 was in 0·2 mM DNP at pH 6·8, the remainder in 2 mM-CN.
Fluxes are calculated with $[Na]_i$ as in Table 1. $k_1$ is the rate constant in artificial sea water at the beginning of the experiment; $k_2$ in CN or DNP just before injecting; $k_3$ was measured at the peak of the response to injection, usually 15 min after injection; $k_4$ after recovery in artificial sea water. The correction factor (which has not been applied) gives the calculated ratio of the total $^{22}Na$ to the quantity which had not diffused beyond the column of ~ P.

sodium. Rate constants after injection, which do not depend on estimates of the internal sodium concentration, are given in Table 3 (Column 7). From Column 9 it will be seen that with high concentrations of arginine phosphate (> 20 mM) the rate constant after injection into a poisoned fibre is about 0·8 of that in the unpoisoned fibre at the beginning of the experiment. However, since the rate constant at the end of the experiment was less than that at the beginning, this comparison is weighted against the injection. Column 10, which gives the ratio of the rate constant after injection to the mean of the initial and final values, probably affords a

fairer basis for comparison; from this it will be seen that with high concentrations of arginine phosphate restoration is about 90% complete. Bearing in mind that the correction for longitudinal diffusion of $^{22}Na$ has not been introduced, the conclusion is that high concentrations of arginine phosphate produce a complete, or very nearly complete, restoration of the sodium efflux. However, the concentrations required to do this are larger than those in the normal fibre; this aspect is considered further on p. 27.

TABLE 4. Rate constants and peak sodium fluxes after injection, nucleotides

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Rate constant ($min^{-1}$/1000) | | | | Extent of restoration | | | |
| Fibre reference | Temperature (°C) | Axon diameter ($\mu$) | Initial concentration (mM ~ P) | Normal, $k_1$ | Poisoned, $k_2$ | Injected, $k_3$ | Normal, $k_4$ | $\frac{k_3}{k_1}$ | $\frac{2k_3}{k_1+k_4}$ | Peak Na efflux after injection (pmole/cm².sec) | Calculated correction factor |
| | | | | | | ATP | | | | | |
| 18 D 6 | 20 | 725 | 2·4 | 2·1 | 0·29 | 0·59 | | 0·28 | 0·38 | 18 | 1·3 |
| 18 D 6 | 20 | 725 | 12·4 | | 0·22 | 1·00 | 1·0 | 0·48 | 0·65 | 30 | 1·4 |
| 20 N 7 | 19 | 890 | 2·8 | 1·4 | 0·26 | 0·69 | — | 0·49 | — | 26 | 1·4 |
| 19 D 6 | 19 | 780 | 5·8 | 2·7 | 0·43 | 1·00 | 1·3 | 0·37 | 0·50 | 32 | 1·4 |
| 19 D 7 | 18 | 911 | 7·4 | 2·2 | 0·41 | 1·27 | — | 0·58 | — | 47 | 1·4 |
| 22 N 7 | 19 | 867 | 8·2 | 1·95 | 0·28 | 1·12 | — | 0·57 | — | 40 | 1·4 |
| 24 O 7 | 18 | 890 | 8·2 | 2·3 | 0·40 | 1·18 | — | 0·51 | — | 40 | 1·3 |
| 27 S 7 | 18 | 850 | 9·0 | 2·2 | 0·12 | 0·95 | — | 0·43 | — | 34 | 1·4 |
| 30 O 7 | 18 | 842 | 9·2 | 1·8 | 0·34* | 0·97* | — | — | — | 46* | 1·4 |
| 10 N 9 | 19 | 776 | 9·5 | 1·5 | 0·21 | 0·97 | 1·0 | 0·66 | 0·79 | 31 | 1·1 |
| 4 O 7 | 17 | 830 | 9·6 | 2·2 | 0·26* | 0·92* | — | — | — | 43* | 1·3 |
| 11 D 6 | 18 | 581 | 12·6 | 3·8 | 0·56 | 1·56 | 1·9 | 0·41 | 0·55 | 38 | 1·3 |
| 7 N 8 | 17 | 805 | 14·3 | 1·5 | 0·35 | 0·90 | 1·0 | 0·60 | 0·72 | 32 | 1·1 |
| 19 N 8 | 17 | 746 | 17·0 | 1·0 | 0·24 | 0·55 | — | 0·55 | — | 17 | 1·2 |
| 12 S 7 | 19 | 578 | 19·6 | 4·2 | 0·59 | 1·60 | 1·6 | 0·38 | 0·5 | 39 | 1·3 |
| 24 N 8 | 16 | 720 | 32 | 1·9 | 0·30 | 0·60 | — | 0·32 | — | 18 | 1·2 |
| | | | | | | ADP | | | | | |
| 11 N 9 | 17 | 806 | 2·1 | 1·5 | 0·28 | 0·54 | — | 0·36 | — | 18 | 1·1 |
| 22 O 7 | 17 | 826 | 16·8 | 2·2 | 0·28 | 0·73 | — | 0·33 | — | 23 | 1·3 |
| 7 N 7 | 18 | 796 | 18·0 | 3·0 | 0·25 | 0·96 | — | 0·32 | — | 29 | 1·4 |
| 16 D 8 | 17 | 606 | 23·1 | 2·2 | 0·38 | 1·2 | — | 0·55 | — | 30 | 1·2 |
| | | | | | | GTP | | | | | |
| 18 N 9 | 16 | 786 | 10·4 | 1·28 | 0·18 | 0·75 | 0·74 | 0·59 | 0·74 | 25 | 1·2 |
| 12 N 9 | 19 | 774 | 10·7 | 2·00 | 0·22 | 0·85 | 0·68 | 0·43 | 0·64 | 27 | 1·1 |
| | | | | | | ITP | | | | | |
| 13 N 9 | 18 | 744 | 11·2 | 1·81 | 0·29 | 0·40 | 1·0 | 0·22 | 0·28 | 12 | 1·1 |

* In K-free solution. 24 O 7 and 30 O 7 were in 0·2 mM DNP at pH 6·8, the remainder in 2 mM-CN. Other details as in Table 3.

*Adenosine triphosphate.* Figure 9 gives the relation between the initial concentration of ATP and the estimated sodium efflux. The efflux appears to rise to a maximum of about 40 pmole/cm².sec at 10 mM ~ P and then to decline at higher concentrations (see Table 4). On p. 581 it is shown that high concentrations of ATP, ADP and pyrophosphate depress the sodium efflux of unpoisoned fibres. This probably accounts for the decline in Fig. 9, but makes it difficult to assess the restorative effect of ATP.

### *The K sensitivity of the Na efflux*

*Arginine phosphate.* In an unpoisoned squid axon removal of external K causes a sudden and reversible decrease in the efflux of sodium. The ratio (Na efflux) $_{\text{K-free}}$:(Na efflux)$_{10\text{mM-K}}$ varied between 0·2 in some fresh fibres to 0·5 or more in axons which had been isolated for long periods; for example, 0·7 in an axon used 24 hr after death of the squid. In the experiments considered here, which were all carried out on axons from refrigerated mantles (p. 562), the average value of the ratio was 0·35; a mean value of 0·30 was obtained on *Sepia* axons (Hodgkin & Keynes, 1955).

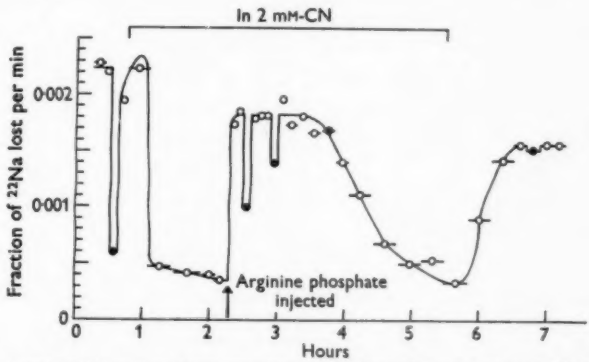


Fig. 10. Effect of injecting a large quantity of arginine phosphate into a CN-poisoned fibre on Na efflux into K-free solution (●) and 10 mM-K (○). The length injected with $^{22}$Na was 10 mm and with arginine phosphate, 15 mm. Immediately after the injection the mean concentration of arginine phosphate in the fibre was 33 mM. Axon 2308, diameter 686 $\mu$, 18° C. At the end of the experiment the action potential was 101 mV.

Since the Na efflux is normally K-sensitive, it was important to find out whether the Na efflux resulting from injections had the usual response to removal of K. An additional reason for carrying out such experiments is that strong inhibitors such as 2 mM-CN or 0·2 mM DNP at pH 6·7 cause a transient rise in the Na efflux into a K-free solution at a time when the Na efflux into 10 mM-K is still near its normal value. This and related effects which will be described in a later paper may be summarized by saying that the K sensitivity of the Na efflux is more susceptible to interference with metabolism than is the absolute level of the Na efflux into normal sea water (Caldwell, Hodgkin, Keynes & Shaw, 1960).

Figure 10 illustrates the effect of injecting a high concentration of arginine phosphate into a fibre poisoned with cyanide on the sodium efflux in 10 mM-K (○) and in a K-free solution (●). In the unpoisoned

fibre the rate constant in 10 mM-K was $2{\cdot}2 \times 10^{-3}$ min$^{-1}$ and removal of K reduced this to $0{\cdot}6 \times 10^{-3}$ min$^{-1}$. After the fibre had been in cyanide for 90 min the rate constant in 10 mM-K was $0{\cdot}4 \times 10^{-3}$ min$^{-1}$; on injecting arginine phosphate (33 mM ~ P) the rate constant in 10 mM-K rose to $1{\cdot}85 \times 10^{-3}$ min$^{-1}$ and remained at this level for an hour. The efflux was initially cut to about a half by removing external K but the K sensitivity decreased fairly rapidly and had disappeared by the end of the plateau. The loss of K sensitivity may be due partly to a loss of arginine phosphate and partly to an accumulation of arginine (see p. 562); an accumulation of arginine might also explain the fact that although the sodium efflux rose

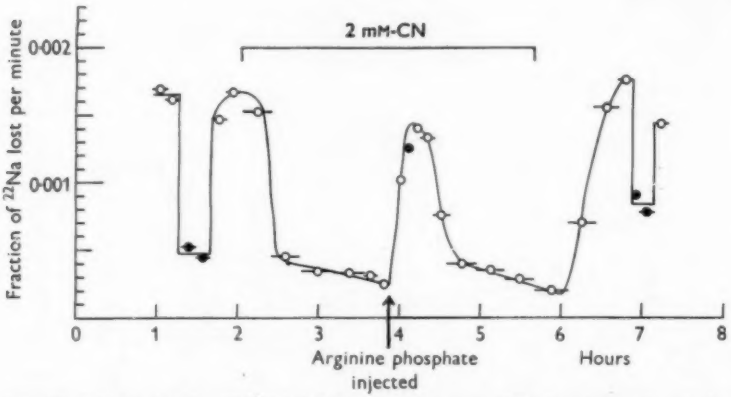


Fig. 11. Similar to Fig. 10 but injecting a smaller quantity of arginine phosphate. The length injected with $^{22}$Na was 10 mm, and with arginine phosphate 15 mm. Immediately after the injection the mean concentration of arginine phosphate in the fibre was 7·4 mM. Axon 7O8, 898 $\mu$, 20° C; final action potential, 99 mV.

to a fairly high level when cyanide was removed there was only a very slight recovery of K sensitivity.

Figure 11 illustrates the action of a dose of arginine phosphate which was about one quarter of that considered previously. In this case the period of enhanced sodium efflux lasted for a relatively short time and there was no plateau. A single observation made near the peak of the curve indicated that the efflux was little affected by removing K. From Table 5 it can be seen that the Na efflux became K-sensitive when the injected arginine phosphate exceeded a concentration in the axon of about 8 mM.

The relation between the concentration of arginine phosphate and the Na efflux into a K-free solution is illustrated by curve *B* in Fig. 8. In contrast to the efflux into 10 mM-K, which remained approximately constant over the range 10–30 mM ~ P, the efflux into a K-free solution probably reached a maximum at about 10 mM ~ P and then declined

slowly as the concentration was increased to 35 mM ~ P. The two curves in Fig. 8 account satisfactorily for the way in which the Na efflux into 10 mM-K and K-free solution vary with time after injection of a large quantity of arginine phosphate. Suppose that, as in the experiment of Fig. 10, the initial concentration of arginine phosphate is 33 mM. As the concentration

TABLE 5. Effect of injections on K sensitivity of sodium efflux

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|
| | | (Na efflux in K-free solution):(Na efflux in 10 mM-K) | | | | |
| Fibre reference | Initial concentration (mM ~ P) | Normal | In CN | In CN soon after injection | In CN long after injection | Normal after recovery |
| | | | Arginine phosphate | | | |
| 7O8 | 7·4 | 0·30 | — | 0·9–1·0 | — | 0·5 |
| 21N8 | 8·5 | 0·41 | — | 1·0 | — | — |
| 17N8 | 11·5 | 0·43 | 1·2 | 0·83 | 1·3 | |
| (17N8 | 45·6 | | 1·3 | 0·68 | — | —) |
| 31O8† | 18 | 0·18† | 1·1† | 0·49† | — | 0·85† |
| 28O8 | 22 | 0·29 | 1·0 | 0·64 | — | 0·78 |
| 27O8* | 23·5 | 0·34 | 1·4* | 0·73* | 1·1 | 1·00* |
| 5N8† | 24 | 0·18† | 1·5† | 0·51† | 1·2† | 0·60† |
| 23O8 | 33 | 0·27 | — | 0·54 | 1·0 | 0·98 |
| 12N8 | 37 | 0·35 | 1·2 | 0·46 | — | 0·86 |
| (19N8 | 43 | 0·31 | 1·4 | 0·51 | — | —) |
| | | | Phosphoenolpyruvate | | | |
| 24N9 | 17 | 0·42 | 1·1 | 0·65 | 1·23 | |
| 26N8 | 20 | 0·30 | 1·4 | 0·68 | — | — |
| (21N8 | 21 | 0·41 | — | 0·81 | — | —) |
| | | | ATP | | | |
| 10N9 | 9·5 | 0·31 | 1·2 | 1·03 | — | 0·69 |
| 7N8 | 14·3 | 0·41 | 1·4 | 0·93 | — | 0·9 |
| 19N8 | 17·0 | 0·31 | 1·4 | 0·94 | — | — |
| 24N8 | 32 | 0·29 | 1·4 | 0·91 | — | — |
| | | | ADP | | | |
| 16D8 | 16 | 0·45 | 1·4 | 1·02 | — | — |
| | | | GTP | | | |
| 18N9 | 10·4 | 0·46 | 1·3 | 1·1 | 1·5 | 0·85 |
| 12N9 | 10·7 | — | — | 1·3 | — | — |

* Axon in K-free solution for most of experiment, 10 mM-K for short periods only. † 20 mM-K instead of 10 mM-K throughout. Results in brackets are second injections of phosphate ester done at a later stage of the experiment. In Columns 5 and 6, 'soon' and 'long' mean about 15 and 120 min, respectively.

falls from 33 to 10 mM the Na efflux into 10 mM-K should remain constant but the efflux into K-free solution should rise steadily. K sensitivity ought to disappear at the end of the plateau. These predictions were borne out by the experiment of Fig. 10 and by other experiments of a similar type.

One point not illustrated by Fig. 10 is that in cyanide either before or long after the injection there is a small effect of K on sodium efflux which is in the opposite direction to that in the normal fibre. In the poisoned fibre removal of K usually increases the residual efflux by 10–40% instead of decreasing the efflux by 60–80% as in a normal fibre. The effect of a maximal injection of arginine phosphate is thus to alter the ratio $(\text{Na efflux})_{\text{K-free}}:(\text{Na efflux})_{10\text{ mM-K}}$ from 1·1–1·4 in cyanide to about 0·5 soon after the injection.

The general conclusion from the experiments with arginine phosphate is that this compound is capable of restoring a K-sensitive efflux of sodium but only when applied at concentrations which are much higher than those in a normal fibre.

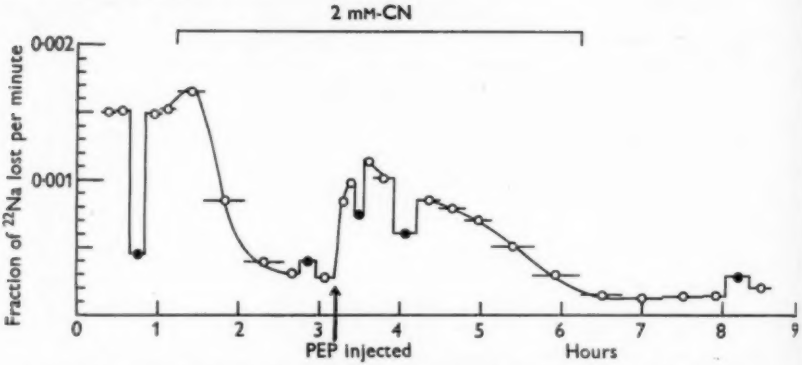


Fig. 12. Effect of injecting phosphoenolpyruvate (PEP) into a CN-poisoned fibre on Na efflux into K-free solution (●) and 10 mM-K (○). The length injected with $^{22}$Na was 10 mm, and with PEP 16 mm. Immediately after the injection the mean concentration of PEP in the fibre was 20·5 mM. Axon 26N8, 860 $\mu$, 17° C; final action potential, 87 mV.

*Phosphoenolpyruvate.* Figure 12 gives the result of injecting phosphoenolpyruvate (PEP) into an axon poisoned with cyanide. The recovery of K sensitivity is definite, but not as marked as with arginine phosphate; the reason may be that the PEP was injected as a sodium salt, for there were indications that the K effect was small when the internal sodium was high.

An unexpected conclusion about the action of PEP, which depends on a single experiment, is that after the response to the injection had worn off there was no final recovery on removing cyanide. This result is illustrated in Fig. 12; the other experiments with PEP, which were terminated in cyanide, provided no evidence about recovery. Since there was always a substantial recovery on removing cyanide after injections of ATP or arginine phosphate, it seems that PEP may give rise to some compound with a blocking action which outlasts removal of cyanide.

*Adenosine triphosphate.* The effect of injecting a large concentration of ATP is illustrated by Fig. 13. The sodium efflux did not rise to such a high value as that obtained after injections of arginine phosphate and it

was not appreciably reduced by removing external K; similar results were obtained in all the other experiments with ATP (Table 5). In one case an injection of arginine phosphate made the Na efflux sensitive to external potassium after a previous injection of ATP had failed to do so.

Although the results of injecting ATP were clearly different from those of injecting arginine phosphate, it is not right to conclude that ATP had no effect at all on K sensitivity. When external K was removed the Na efflux of a fibre in cyanide normally increased, instead of decreasing as in an unpoisoned fibre. After an ATP injection removal of K no longer increased the Na efflux, so that in this sense the ATP did have a small effect on K sensitivity.

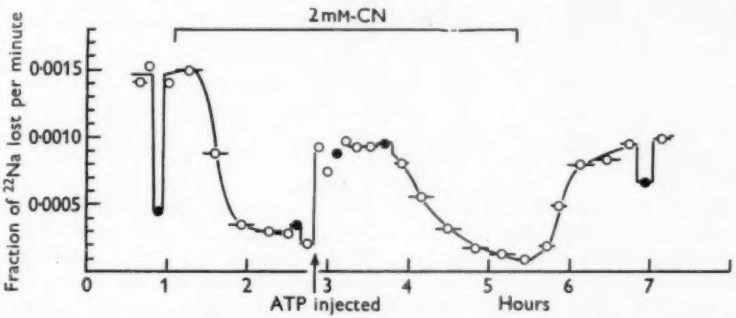


Fig. 13. Effect of injecting ATP into a CN-poisoned fibre on Na efflux into K-free solution (●) and 10 mM-K (○). The length injected with $^{22}$Na was 12 mm and with ATP, 18 mm. Immediately after injection the mean concentration of ATP in the fibre was 4·8 mM (9·5 mM ~ P). Axon 10N9, 776 $\mu$, 19° C; final action potential, 92 mV. The ATP contained about 4 % of the phosphorus as ADP.

*Adenosine diphosphate, guanosine triphosphate and inosine triphosphate.* These compounds increased the Na efflux of a cyanide poisoned fibre but did not restore the normal K sensitivity (Table 5). The Na efflux after a GTP injection resembled the residual efflux in cyanide in being increased by removing K instead of being reduced as in the normal fibre.

### *The effects of injections on Na efflux in unpoisoned fibres*

*Arginine phosphate.* Injections of arginine phosphate into normal fibres had little effect on Na efflux or on its K sensitivity. Tests were made with external potassium concentrations of 0, 10, and 20 mM; in no case was there any appreciable change in Na efflux after the injection of arginine phosphate. These results suggest that the normal fibre contains enough arginine phosphate to saturate the transport mechanism.

Since the K sensitivity of the sodium efflux declined when fibres were left for 12–24 hr in sea water, it seemed possible that arginine phosphate might have a beneficial effect when injected into an old fibre. This point was tested on one fibre which was used 15 hr after the death of the squid.

Figure 14 shows that injection of arginine phosphate affected neither the K sensitivity nor the absolute level of the sodium efflux. The conclusion is that the low K sensitivity of old fibres does not depend on a low concentration of arginine phosphate but on some other change.

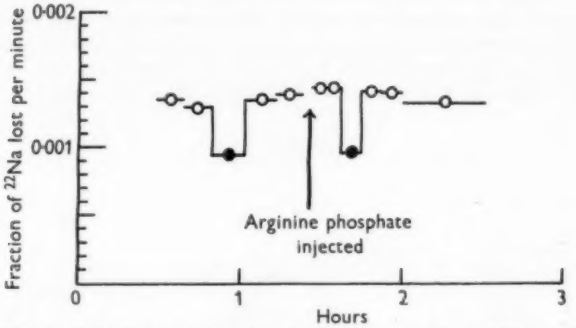


Fig. 14. Effect of injecting arginine phosphate into an unpoisoned fibre on Na efflux into K-free solution (●) and 10 mM-K (○). The length injected with $^{22}$Na was 10 mm and with arginine phosphate, 16 mm. Immediately after injection the mean concentration of the injected arginine phosphate in the fibre was 37 mM. Axon 14N8, 805 $\mu$, *ca.* 18° C. This axon, which was taken from a squid which had just died, was kept for 15 hr, mainly at 0° C, before use. The action potential was 104 mV at the beginning of the experiment and 102 mV at the end.

*Adenosine triphosphate.* When injected in fairly high concentrations (> 3 mM), ATP depressed the efflux of sodium from an unpoisoned fibre. Figure 15*B* illustrates the decrease in efflux resulting from an injection which gave an initial rise of 8 mM (16 mM ~ P). The effects of high concentrations of pyrophosphate were similar to those of ATP (Fig. 15*A*) A single experiment showed that ATP at a concentration in the axon of 5 mM depressed the efflux into K-free solution in about the same proportion as that into 10 mM-K. High concentrations of ATP seem to depress the absolute level of the Na efflux but not to alter its K sensitivity. As the depressant action of ATP was reminiscent of the inhibitory effect of an excess of ATP on the adenosine triphosphatase of myofibrils (Perry & Grey, 1956), the effects of Mg were investigated. Introduction of Mg (as $MgCl_2$) in an amount equivalent to the ATP appeared to have a protective effect, neither the Mg nor the subsequent dose of ATP causing any marked change.

*Adenosine diphosphate.* Figure 16 shows the result of injecting sufficient ADP to raise the concentration in the axon by 2·6 mM. The effect of ADP was to raise the Na efflux into K-free sea water and to cause a slight reduction in the efflux into 10 mM-K. In contrast to ATP, ADP reduces K sensitivity.

With a much larger quantity of ADP (13 mM in the axon) the injection left the Na efflux into K-free sea water unaltered and reduced the efflux into 10 mM-K to the K-free level. Apparently a high concentration of ADP has an inhibitory effect which is similar to that of excess ATP.

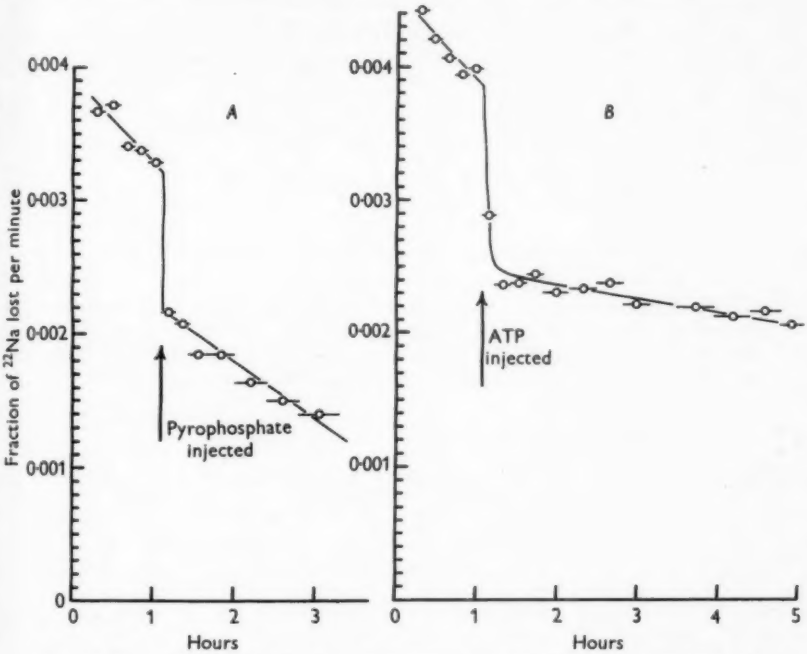


Fig. 15. The effect on Na efflux from unpoisoned axons of injecting pyrophosphate and ATP. In both experiments the axons were loaded with a 12 mm column of $^{22}$Na, and the phosphate compounds were injected over the same 12 mm. *A*: axon 17S7, diameter 658 $\mu$, temperature 18° C, concentration of potassium pyrophosphate inside axon after injection was 14·4 mM. *B*: axon 16S7, diameter 640 $\mu$, temperature 18·5° C, concentration of K salt of ATP inside axon after injection was 8·0 mM (16 mM ~ P).

*Arginine.* In fibres poisoned with cyanide a high concentration of arginine phosphate increases the K sensitivity of the sodium efflux. One might therefore expect that a high concentration of arginine would reduce the K sensitivity of the normal efflux. Figure 17 shows that the initial effect of injecting arginine into an unpoisoned fibre was to increase the Na efflux into K-free solution. Before injecting arginine the ratio $(\text{Na efflux})_{\text{K-free}} : (\text{Na-efflux})_{10\,\text{mM-K}}$ was 0·23; 5 min after the injection it was 0·8. On leaving the fibre there was a slow recovery in K sensitivity and a final injection of arginine phosphate caused only a slight improvement.

The slow recovery is not understood, since there was not enough phosphate in the axon to convert much of the arginine into arginine phosphate.

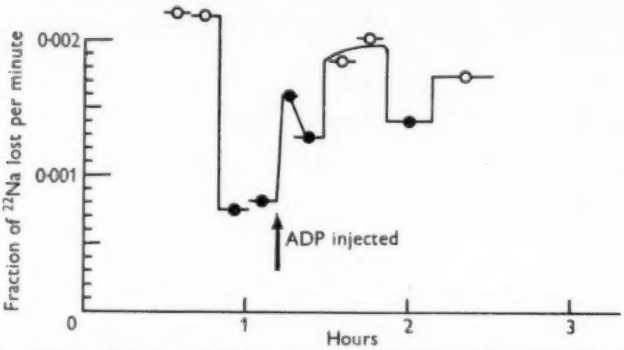


Fig. 16. Effect of injecting ADP into an unpoisoned fibre on Na efflux into K-free solution (●) and 10 mM-K (○). The length injected with $^{22}$Na was 10 mm and with ADP, 16 mm. Immediately after injection, the mean concentration of ADP in the fibre was 2·6 mM. Axon 27N8, 860 $\mu$, 19° C; final action potential, 80 mV.

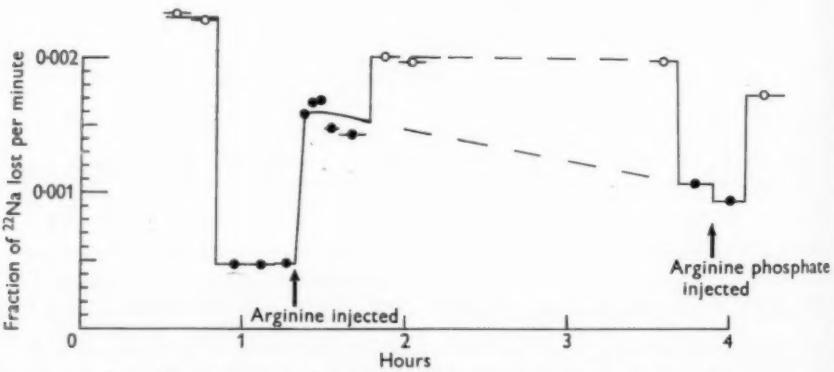


Fig. 17. Effect of injecting arginine HCl into an unpoisoned fibre on Na efflux into K-free solution (●) and 10 mM-K (○). The length injected with $^{22}$Na was 10 mm and with arginine HCl 16 mm. Immediately after injection the mean concentration of arginine in the fibre was 62 mM. Axon 9D8, 788 $\mu$, 18° C, final action potential 92 mV.

*The effect of injections on the other Na and K fluxes in poisoned fibres*

*Na influx: the effect of CN and of ATP injections.* The experiments on Na influx were done before the difference between the effects of ATP and of arginine phosphate on the Na efflux had been appreciated, and were confined to the action of injected ATP. The Na influx was determined by exposing the axon to a solution containing $^{24}$Na for 10 min, and then taking

counts for about an hour with a stream of inactive sea water flowing past the axon. The removal of adherent small fibres and connective tissue from the giant axon was done as completely as possible, but even so about 40 min elapsed before all the superficial $^{24}Na$ was washed away. However, after this period the radioactivity declined with a low rate constant of the same order (0·01–0·02 $hr^{-1}$) as that found for CN-treated axons injected

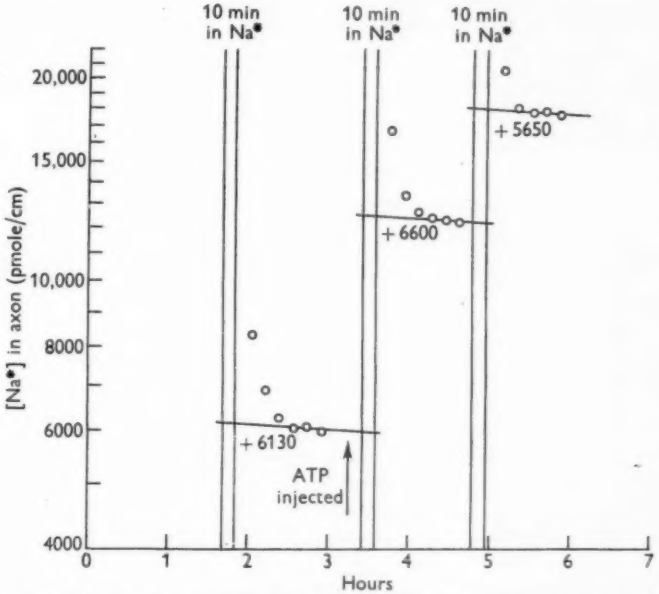


Fig. 18. The effect of injecting ATP on the influx of labelled sodium. Both the $^{24}Na$ solution and the inactive solution in which counts were taken contained 2 mM-CN, and the axon had been in the CN-sea water for 2·2 hr before the first period in $^{24}Na$. Sufficient ATP was injected at the arrow to give a mean concentration of 5·3 mM in the axoplasm, over a length of 20 mm. Axon 9D7, diameter 768 $\mu$, temperature 17° C. At the end of experiment the resting potential was 58 mV, action potential 97 mV.

with $^{22}Na$, and could therefore be taken to represent labelled Na which had penetrated into the axoplasm. In earlier work with a similar technique (see Hodgkin & Keynes, 1955) it was easier to distinguish between extracellular and intracellular radioactivity, since the $^{24}Na$ was introduced by stimulation, and relatively more entered the interior of the axon. Clearly, it would be desirable to make more measurements with extruded axoplasm in order to avoid this difficulty.

One experiment is illustrated in Fig. 18 and the results of this and four others are listed in Table 6. It appears that, as in DNP-treated *Sepia*

axons (Hodgkin & Keynes, 1955), the Na influx was little changed by poisoning with 2 mM-CN (8 unpublished experiments with smaller axons which were done in 1952 gave an average resting Na influx into unpoisoned squid axons of 49 pmole/cm².sec; Shanes & Berman (1955) give 62 pmole/cm².sec for the axons of *L. pealii*). For what it is worth, the table suggests that there may be a slight increase in Na influx immediately after the injection of ATP, which would be qualitatively consistent with the idea that ATP promotes an exchange diffusion of Na across the membrane. But the action of ATP on the influx seems to be definitely less than its stimulating effect on the efflux.

TABLE 6. The effect of injecting ATP on the influx of labelled sodium into CN-poisoned axons

| Axon diameter (μ) | Influx before injection (pmole/cm².sec) | Influx 15 min after injection (pmole/cm².sec) | Influx 1·5 hr after injection (pmole/cm².sec) | [ATP] after injection (mM ~ P) |
|---|---|---|---|---|
| 850 | 35 | 21 | — | 8·6 |
| 880 | 33 | 71 | 38 | 8·0 |
| 768 | 42 | 45 | 39 | 10·6 |
| 856 | 48 | 50 | 54 | 8·6 |
| 935 | 53 | 72 | 37 | 7·2 |
| Mean | 42 | 52 | 42 | 8·6 |

All the axons had been treated with 2 mM-CN for at least 1·2 hr before making the first influx measurement. In the first experiment the Na influx before poisoning was determined as 32 pmole/cm².sec; in the other experiments the unpoisoned influx was not measured. Temperature 17–19° C.

*K efflux: the effect of CN and of ATP injection.* Figure 19 shows that neither cyanide nor a subsequent injection of ATP had any marked effect on the efflux of labelled potassium. A second experiment on similar lines gave the same result. This emphasizes the difference in nature between the passive K efflux, effectively independent of metabolism, and the metabolically-driven Na efflux.

*K influx: effects of cyanide and of injecting ATP or arginine phosphate.* Determinations of K influx by counting intact squid axons proved to be complicated by the presence of substantial amounts of K in a superficial region of the fibre which exchanged much more rapidly than K introduced into the axon by micro-injection. It was therefore necessary to rely on measurements with extruded axoplasm, pairs of axons being used wherever possible. These results are given in Table 7. Tentative conclusions are (1) cyanide reduces K influx to about 25–50%; (2) after cyanide poisoning, injections of ATP, in concentrations giving about the maximum Na efflux, may give an increase in K influx—however, this increase was

small and was not statistically significant; (3) after cyanide poisoning, injections of arginine phosphate increase K influx about threefold.

These results are consistent with the idea that injections of arginine phosphate are more effective than ATP in restoring the coupling between K entry and Na exit. However, it now seems unlikely that there can be a

TABLE 7. K influxes in paired axons

I. Effect of CN (uncleaned axons)

| Diameter (μ) | | K influx (pmole/cm².sec) | | |
|---|---|---|---|---|
| | | 2 mM-CN | Unpoisoned | Difference |
| *A* | *B* | *A* | *B* | *B* − *A* |
| 638 | 638 | 5·8 | 20·7 | 14·9 |
| 712 | 690 | 13·6 | 14·8 | 1·2 |
| 622 | 645 | 8·7 | 21·6 | 12·9 |
| Mean ± S.E. | | 9·3 ± 2·3 | 19·0 ± 2·2 | 9·7 ± 4·1 |

II. Effect of ATP after CN (cleaned axons)

| Diameter (μ) | | | | | |
|---|---|---|---|---|---|
| | | | 2 mM-CN, injected with ATP | | Concentration of ATP in axon (mM ~ P) |
| *A* | *B* | 2 mM-CN | | Difference | |
| 955 | 986 | 6·6 | 9·1 | 2·5 | 6·8 |
| 655 | 684 | 3·6 | 4·4 | 0·8 | 14 |
| 850 | 924 | 2·6* | 8·0* | 5·4* | 7·2 |
| Mean ± S.E. | | 4·3 ± 1·2 | 7·2 ± 1·4 | 2·9 ± 1·3 | |

III. Effect of arginine phosphate after CN (cleaned axons)

| Diameter (μ) | | | | | |
|---|---|---|---|---|---|
| | | | 2 mM-CN, injected with arginine phosphate | | Concn. of arginine phosphate in axon (mM) |
| *A* | *B* | 2 mM-CN | | Difference | |
| 681 | 709 | 5·0 | 14·3 | 9·3 | 48 |
| 761 | 756 | 4·3 | 12·9 | 8·6 | 42 |
| 841 | 810 | 4·4 | 12·5 | 8·1 | 37 |
| 806 | 821 | 3·2 | 14·1 | 10·9 | 35 |
| 844 | 831 | 4·6 | 14·6 | 10·0 | 35 |
| Mean ± S.E. | | 4·3 ± 0·3 | 13·7 ± 0·4 | 9·4 ± 0·5 | |

* These axons were stimulated at 156/sec for 20 min before being placed in cyanide.

ATP or arginine phosphate was injected over a length of 20 mm about 10 min before a 10 min exposure to $^{42}K$. After removal from $^{42}K$, axons were soaked 10 min in inactive sea water before extruding axoplasm; in the case of injected axons, axoplasm was taken from 15 mm in the injected region. Control and injected axons were kept for the same period in CN (1½–2 hr). Temperature, 17–19° C. The mean resting influx in unpoisoned cleaned axons was 16·3 ± 2·2 pmole/cm².sec (5 experiments).

one-to-one coupling between K entry and Na exit. From Table 7 the Na efflux resulting from an injection of arginine phosphate is probably about 50 pmole/cm².sec. Since the Na efflux is reduced to about 25 pmole/cm².sec on removing K one might expect a K influx of 25 pmole/cm².sec if the K effect depends on a component of the Na efflux which is coupled in a one-to-one fashion with K influx. In practice, the extra

K influx resulting from an injection of arginine phosphate was about 10 pmole/cm².sec. Both this result and those obtained with normal fibres are better fitted by a theory in which an entry of one $K^+$ ion promotes the exit of 2 or 3 $Na^+$ ions.

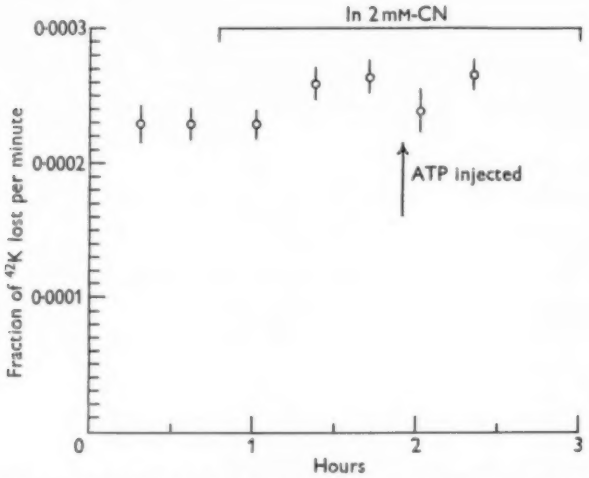


Fig. 19. The effect of CN and injection of ATP on the potassium efflux from a squid axon. The axon was loaded with $^{42}K$ by the injection of a 13 mm column of 0·57 M-K*Cl at zero time. At the arrow, sufficient ATP was injected to give a mean concentration of 8 mM ~ P over the same 13 mm. The lengths of the vertical lines indicate $\pm 2 \times$ S.E. of the counts. Axon 11N7, diameter 912 $\mu$, temperature 17° C. At end of experiment resting potential was 59 mV, action potential 108 mV.

## DISCUSSION

The object of this investigation was to see how far the effects of metabolism on the movements of Na and K could be imitated by injecting substances like arginine phosphate and ATP. The general answer is that both compounds increase the Na efflux of a fibre poisoned with cyanide or DNP, but that injected arginine phosphate is a more effective substitute for metabolism than injected ATP. Thus, arginine phosphate restores the normal coupling between Na efflux and K influx, whereas injected ATP seems to have little effect on K influx and gives a Na efflux which is not K sensitive. Of the other compounds containing ~ P which were tested, only phosphoenolpyruvate gave a Na efflux which was reduced by removing external K; creatine phosphate caused no increase and ITP a very slight increase in Na efflux; ADP and GTP caused a substantial increase in efflux which was not reduced by removing external K.

The compounds which increase Na efflux might all do so by providing a supply of ATP at the membrane. For ATP itself no intermediate reaction is required; for the other three compounds in which we were principally interested the reactions and enzymes might be as follows:

I. ADP + arginine P = ATP + arginine (Arginine phosphokinase),

II. ADP + pyruvate P = ATP + pyruvate (Pyruvate phosphokinase),

III. 2 ADP = ATP + AMP (Adenylate kinase).

In this hypothesis it is assumed that the axoplasm contains the necessary enzymes and that a small amount of ADP is left after cyanide poisoning. Evidence for these assumptions is provided by recent unpublished observations which show that injection of arginine phosphate into fibres poisoned with cyanide does regenerate ATP from AMP and that injection of phosphopyruvate under similar conditions regenerates both ATP and arginine phosphate. Some further assumption is needed to explain the effects of arginine phosphate and phosphoenolpyruvate in making the sodium efflux sensitive to removal of potassium. One possibility is that the transport system requires for its normal operation not only a supply of ATP but also that there should be a high ATP:ADP ratio near the membrane. On this view, the effects of arginine phosphate or phosphopyruvate on the coupling between K and Na movements arise from their action in keeping ADP at a low level by reactions I and II; ATP does not restore K sensitivity because it rapidly produces ADP by reaction III. The results of injecting ADP or arginine into unpoisoned fibres were consistent with this idea, since both substances increased the Na efflux into a K-free solution without having much effect on the efflux into a solution containing 10 mM-K. However, such experiments do not prove that the conversion of ATP to ADP is the reaction immediately concerned with ionic transport. It could equally well be argued that the transporting mechanism requires a high ratio of arginine phosphate to arginine, and that ADP affects the coupling between K and Na movements by converting arginine phosphate to arginine. Indeed, if the various forms of high-energy phosphate are in rapid equilibrium, it may be very difficult to decide which phosphate compound is most directly concerned in driving ion transport.

A quantitative difficulty raised by the experiments is that although injected arginine phosphate gave a complete, or nearly complete, restoration of the normal sodium efflux, the concentration required was about twice that in a normal fibre. Still higher concentrations were needed to make the Na efflux K sensitive. Two possible causes of the discrepancy will be considered. In the first place, the figure for the concentration of arginine phosphate was obtained by dividing the quantity injected per centimetre

by the cross-sectional area of the axon. The peak sodium efflux was reached 10–15 min after the injection and not instantaneously as would be expected if radial diffusion of arginine phosphate were so rapid that it could be neglected. By the time the Na efflux reaches its peak some arginine phosphate has probably broken down, so the total quantity in the fibre should be less than the amount injected. Furthermore, the concentration at the surface of the axon should be less than the mean concentration, since there must be a diffusion gradient in the axoplasm. Calculations with an equation in Carslaw & Jaeger (1947, p. 308, eqn. 7) suggest that with a diffusion coefficient of $0{\cdot}4 \times 10^{-6}$ cm$^2$/sec the surface concentration of arginine phosphate would reach a maximum in about 15 min and that the maximum surface concentration would be only one third of the initial mean concentration. A similar argument should hold if the diffusion coefficient of arginine phosphate in axoplasm is not as low as $0{\cdot}4 \times 10^{-6}$ and the delay in the rise of Na efflux is due to an intermediate chemical reaction.

Another explanation of the quantitative discrepancy is that the poisoned fibre probably contains an abnormal amount of break-down products such as arginine, AMP or inorganic phosphate. In the presence of these compounds it may require an unusually large concentration of arginine phosphate to restore a situation approaching that in the normal fibre.

The same arguments might also explain why very high concentrations of arginine phosphate are needed to restore the K sensitivity of the Na efflux. It seems likely that the loss of K sensitivity depends on the accumulation of a break-down product which may perhaps be identified with ADP. Possibly only a very slight rise in concentration, say from 0·01 to 0·1 mM, may be enough to abolish K sensitivity. In that case significant amounts of ADP might accumulate near the membrane unless the mitochondria in the immediate vicinity were metabolizing normally. Very large concentrations of arginine phosphate might then have to be injected into poisoned fibres in order to reduce the ADP at the surface to the normal level.

## SUMMARY

1. Injections of adenosine triphosphate or diphosphate, guanosine triphosphate, arginine phosphate and phosphoenolpyruvate (PEP) caused a transient increase in the efflux of labelled sodium from squid giant axons which had been poisoned with 2 mM cyanide. Injections of creatine phosphate, adenosine monophosphate, arginine, inorganic phosphate, pyrophosphate and various salt solutions had no restorative action on fibres poisoned with cyanide; inosine triphosphate had only a very slight effect.

2. Injections of ATP and arginine phosphate also had a restorative effect on fibres poisoned with 0·2 mM DNP at pH 6·7; the other compounds were not tested.

3. ATP and arginine phosphate had no effect when applied externally to fibres poisoned with cyanide.

4. The duration of the response to ATP or arginine phosphate varied between about half an hour with a small quantity to several hours when injecting 5–10 times the quantity in an unpoisoned fibre.

5. The number of Na ions extruded was roughly proportional to the number of molecules of phosphate ester injected. Na: $\sim$ P ratios were about 0·7 for ATP, arginine phosphate and PEP, about 0·4 for ADP and GTP and about 0·03 for ITP.

6. The Na effluxes obtained by injecting high concentrations of arginine phosphate into poisoned fibres were intermediate between the levels in unpoisoned fibres at the beginning and end of the experiment.

7. The Na effluxes resulting from injections of ATP, ADP or GTP differed from the normal efflux in that they were not reduced by removing external potassium. On the other hand, the Na effluxes resulting from injections of arginine phosphate or PEP resembled the normal efflux in that initially they were substantially reduced by removing external potassium.

8. Injections of arginine phosphate increased the K influx of cyanide-poisoned fibres about threefold; ATP injections had relatively little effect.

9. The downhill fluxes (Na influx and K efflux), which were little altered by cyanide, were not much changed by injecting ATP into fibres poisoned with cyanide.

10. The Na efflux of unpoisoned axons was not affected by injections of arginine phosphate and was depressed by high concentrations of ATP, ADP or pyrophosphate. Injections of ADP or arginine made the Na efflux of the normal fibre insensitive to removal of external potassium.

11. The experiments provide further support for the view that metabolism drives the cation transport system by generating high-energy phosphate bonds which are carried to the membrane by substances like arginine phosphate and ATP. They also suggest that the presence of arginine phosphate as well as ATP may be required for the normal operation of the active transport mechanism.

We wish to thank the Director and Staff of the Laboratory of the Marine Biological Association, Plymouth, for assistance and for the time spent in collecting squid. We are also indebted to Professor Ennor for a gift of arginine phosphate and to the Royal Society, the Rockefeller Foundation and the Nuffield Foundation for financial assistance.

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# PARTIAL INHIBITION OF THE ACTIVE TRANSPORT OF CATIONS IN THE GIANT AXONS OF *LOLIGO*

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It has been known for several years that the outward movement of sodium from the giant axons of squid and cuttlefish can be greatly reduced either by removing potassium ions from the external medium or by applying metabolic inhibitors such as cyanide or dinitrophenol (Hodgkin & Keynes, 1955). In the earlier work the effects of removing potassium or of inhibitors were studied separately and inhibitors were usually applied in solutions which had the normal potassium concentration of 10 mM. This paper is concerned with the rather curious results obtained by measuring the effects of inhibitors on the efflux of sodium into a potassium-free solution. If the normal sodium efflux is first reduced by removing K, the initial effect of a strong inhibitor, such as 2 mM-CN or 0·2 mM DNP at pH 6·7, is a transient rise in the efflux of sodium into a K-free solution; this lasts for 30–60 min, after which the efflux falls to the low value characteristic of a fully poisoned axon. With a milder inhibitor, such as 0·2 mM DNP at pH 8, there is a maintained rise in the Na efflux into a K-free solution. Taken by themselves these results are puzzling, but they fit well with the observations that arginine phosphate breaks down before adenosine triphosphate (ATP) (Caldwell, 1960) and that injecting arginine phosphate into poisoned fibres restores a K-sensitive sodium efflux, whereas injecting ATP produces a K-insensitive sodium efflux (Caldwell, Hodgkin, Keynes & Shaw, 1960). In more general terms it seems that mild or partial inhibition of metabolism may increase the efflux of sodium into a K-free solution by uncoupling Na and K movements.

## METHODS

Except where indicated the apparatus and method were the same as in the preceding paper (Caldwell *et al.* 1960).

## RESULTS

### *The effect of applying 2 mM-CN in the presence or absence of external potassium*

The full curve and the filled circles in Fig. 1 illustrate the action of cyanide on the efflux of sodium into a K-free medium. To begin with, the axon was in normal sea water containing 10 mM-K and 460 mM-Na. On changing to a similar K-free solution containing 460 mM-Na, the Na efflux dropped suddenly to about one fifth of its previous value. When the solution was changed to a K-free cyanide solution, the Na efflux rose gradually for about 20 min and then declined in 1–2 hr to a level which was somewhat less than the original, unpoisoned value in K-free sea water. The broken

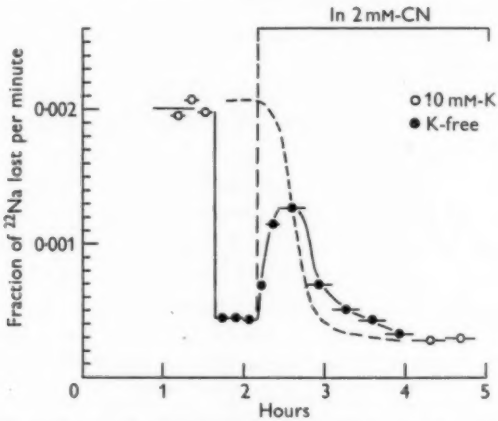


Fig. 1. The effect on Na efflux of applying 2 mM-CN in a K-free medium. Axon diameter 650 $\mu$, temperature about 19° C. The dashed line shows the decline of the efflux with a normal external (K); it was obtained by averaging all the experiments carried out in 1956 and 1957 with 10 mM-K and 2 mM-CN.

curve, which was obtained by averaging all the experiments carried out in 1956 and 1957, shows the effect of cyanide on the efflux of sodium into a solution containing 10 mM-K. With this solution there was usually no initial rise on applying cyanide, and after a delay of 10–20 min the efflux gradually declined to its fully poisoned level. The two curves in Fig. 1 are not strictly comparable, since they were not obtained on the same fibre and since the rate at which energy-rich phosphate disappears may vary with the potassium concentration of the external medium. In order to find out when the Na efflux ceases to be K-sensitive it is necessary to make measurements of the type shown in Fig. 2.

There was some variation in the initial effects of cyanide. The

experiments carried out in 1957 all gave results very similar to those in Fig. 1. However, a further set of experiments done in 1958 showed a larger transient rise in the Na efflux into a K-free solution and on some occasions a small transient rise in the efflux into 10 mM-K. Figure 2 illustrates one of the later experiments. In this case alternate measurements with 10 mM-K and K-free solution were made during the period when cyanide was having its effect; curves for the efflux into the two solutions can therefore be given for the same axon.

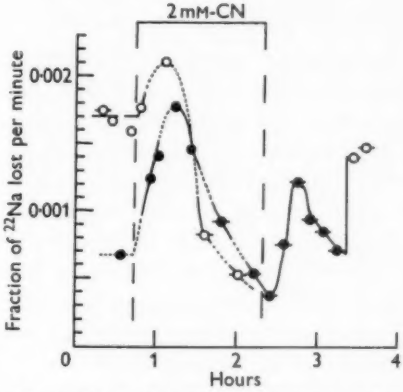


Fig. 2. The effect of 2 mM-CN on Na efflux in solutions containing 10 mM-K (○) or K-free (●). Axon diameter 830 $\mu$, temperature 20° C. The curves are broken during the period when the axon was in the other solution.

Although there are minor differences between Figs. 1 and 2, both show that the early effects of cyanide are a rise in the Na efflux into a K-free solution and a loss in the K sensitivity of the Na efflux; later, the effluxes into 10 mM-K and into K-free solution both decline to a low value. In three experiments, K sensitivity was lost after 30–40 min in cyanide, at which time the Na efflux was 50–80 % of the normal efflux in 10 mM-K. A further reduction to 10–20 % occurred within about 1·5 hr after applying cyanide. If these times are compared with Caldwell's (1960) observations on the disappearance of phosphate esters in cyanide it will be seen that the K sensitivity of the Na efflux is lost at about the time when arginine phosphate disappears from the axoplasm, and that the final reduction of the Na efflux corresponds roughly with the time taken for all the ATP to break down in cyanide.

The latter part of Fig. 2 shows that there was a transient rise in the Na efflux into a K-free solution when cyanide was removed as well as when it was applied. This result is to be expected, since it is likely that ATP is resynthesized before arginine phosphate.

*The effect of Na efflux of* 0·2 mM *DNP at pH* 6·7

With this inhibitor the break-down of arginine phosphate and ATP in squid axons follows much the same course as in 2 mM-CN (Caldwell, 1960). When the inhibitor was applied under K-free conditions there was a transient rise in Na efflux of the same kind as in Fig. 1. As with 2 mM-CN, the rise in efflux occurred during the period when the fibre contained ATP but not arginine phosphate.

*The effect on Na efflux of* 0·2 mM *DNP at pH* 8

If 0·2 mM DNP is applied at a pH of 8 the final concentration in the axoplasm is about one seventh of that found when the inhibitor is applied at pH 6·7 (Caldwell, 1960). The probable reason is that DNP is a weak acid and that the undissociated compound crosses the membrane more readily than the anion. When 0·2 mM DNP is applied at pH 6·7 both arginine phosphate and ATP disappear; at pH 8, arginine phosphate disappears but there is little break-down of ATP. Application of 0·2 mM DNP at pH 8 produced a sustained effect on the Na efflux, similar to the peak of the transient effect of stronger inhibitors such as 2 mM-CN or 0·2 mM DNP at pH 6·7. With 10 mM-K in the external solution there was only a slight reduction in Na efflux, but, in the absence of K, 0·2 mM DNP at pH 8 caused the Na efflux to rise to a steady level which was maintained for several hours. Figure 3 illustrates one of the experiments. To begin with, removing K from the solution outside the unpoisoned fibre reduced the rate constant to about one fifth. On applying 0·2 mM DNP at pH 8, the efflux into the K-free solution rose to about two-thirds of its normal value and then remained fairly steady for 1½ hr. At the end of this time the efflux was K-insensitive, as is shown by the absence of any increase when (K) was raised from 0 to 10 mM. When 0·2 mM DNP was removed there was no change in the efflux into 10 mM-K, but the efflux into K-free solution decreased as the transport system once more became K-sensitive.

The experiments with 0·2 mM DNP at pH 8 showed that the effects of the inhibitor were, first, to produce little change in the Na efflux into 10 mM-K; secondly, to increase the Na efflux into K-free solution and thirdly, to reduce or abolish the K sensitivity of the Na efflux. If disappearance of arginine phosphate is responsible for these changes it should be possible to restore the normal situation by injecting arginine phosphate. Figure 4 shows that an injection of arginine phosphate into an axon poisoned with 0·2 mM DNP at pH 8 caused a transient restoration of K sensitivity. The recovery, which lasted for 30–60 min, took the form of a reduction of the Na efflux into the K-free solution without much change in the efflux into 10 mM-K. The K sensitivity was not restored to

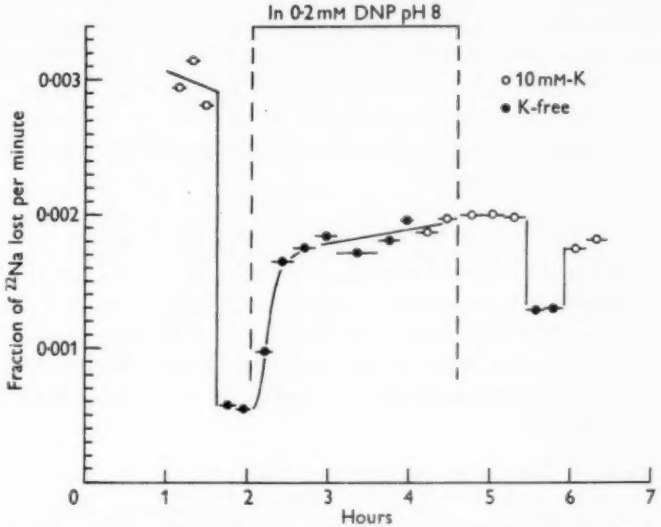


Fig. 3. The effect on Na efflux of applying 0·2 mM DNP at a pH of about 8 in a K-free medium. Axon diameter 735 $\mu$. Temperature 18·5° C.

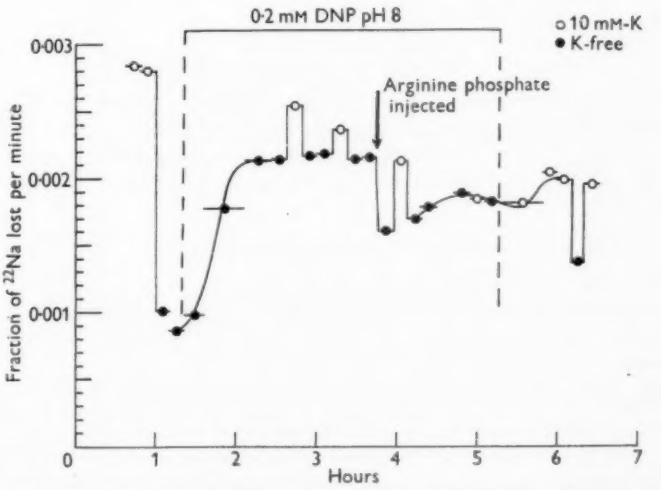


Fig. 4. The effect on Na efflux of injecting arginine phosphate into a fibre poisoned with 0·2 mM DNP at pH 8. A length of 10 mm was injected with $^{22}$Na and a length of 14 mm was injected with arginine phosphate, there being an overlap of 2 mm at either end. The mean initial concentration of arginine phosphate in the axoplasm immediately after the injection was 10 mM. Axon diameter, 783 $\mu$; temperature 18° C.

the original level, but this could hardly be expected since there was only a partial recovery when DNP was removed at the end of the experiment.

Injections of ATP into fibres poisoned with 0·2 mM DNP at pH 8 had little effect on the sodium efflux and did not restore K sensitivity. This is reasonable, since the partially poisoned fibres should have retained ATP and since injections of ATP are not sufficient to restore K sensitivity to fibres poisoned with cyanide.

### *The effect of* 0·2 mM *DNP at pH* 8 *on K influx*

Since 0·2 mM DNP at pH 8 seems to interfere with the normal coupling between Na and K movements it might be expected to reduce the influx of potasssium. The experiments in Table 1, which were carried out by measuring the amount of $^{42}K$ in the axoplasm extruded from paired axons, show that the influx of potassium was halved by exposure to 0·2 mM DNP at pH 8 for 1½–2 hr.

TABLE 1. Effect of 0·2 mM DNP at pH 8 on K influxes in paired axons

| Diameter | | K influx (pmole/cm².sec) | |
|---|---|---|---|
| *A* | *B* | 0·2 mM DNP, pH 8 | Unpoisoned, pH 8 |
| 765 | 782 | 10·2 | 20·2 |
| 730 | 686 | 11·2 | 22·4 |
| 769 | 778 | 7·0 | 17·4 |
| 980 | 958 | 17·7 | 34·8 |
| 873 | 767 | 10·1 | 23·9 |
| Mean ± S.E. | | 11·2 ± 1·8 | 23·7 ± 2·9 |

The axons, which were not cleaned, were exposed to 0·2 mM DNP at pH 8 in artificial sea water for 2–3 hr. During the last 20–30 min of exposure the solutions contained $^{42}K$. At the end of that time axoplasm from the central 20 mm of the axon was extruded. Temperature 18° C.

### *The effect of anoxia on Na efflux*

The effect of anoxia on sodium efflux was examined in two experiments. After injecting $^{22}Na$ the fibre was placed in a stoppered glass vessel containing artificial sea water through which air or nitrogen was bubbled. The sea water round the fibre could be removed through glass taps for counting or replenished without admitting air.

The first experiment which was carried out with 10 mM-K in the external solution confirmed Shanes & Berman (1955) in showing that anoxia reduced the efflux of sodium into a solution containing the normal amount of K. The efflux was reduced to 30–40 %, but as there is likely to be some variation between axons little weight should be attached to a single observation.

The second experiment, which was carried out in a K-free solution, gave a result very similar to that obtained with 0·2 mM DNP at pH 8 (Fig. 3). Thus the sodium efflux, which was first reduced by removing K in aerated sea water, rose when oxygen was replaced by nitrogen and remained at a relatively high value until air was again admitted. The observation that the effect of nitrogen resembles that of 0·2 mM DNP at pH 8, rather than that of 0·2 mM DNP at pH 6·7 or 2 mM-CN, suggests either that the method of removing oxygen

was inadequate or that 2 mM-CN may block an anaerobic pathway as well as the oxidative system of the axon. According to Lohmann (1936) octopus muscle has only a feeble glycolytic system but there is no information about the extent to which cephalopod nerve can obtain energy by glycolysis. Further experiments on the effects of anoxia and of different concentrations of cyanide are plainly needed.

### *The effect on Na efflux of replacing external Na by Li*

The object of these experiments was to see whether there was any evidence that the K-insensitive component of the sodium efflux might consist of an exchange of sodium across the membrane. It had been shown

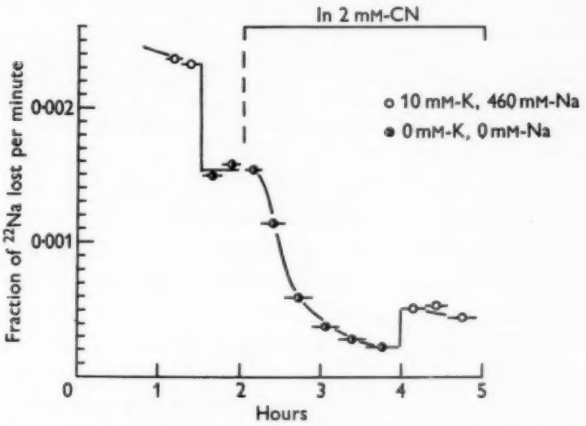


Fig. 5. The effect of applying 2 mM-CN when the external medium contained neither K nor Na. Li was used as the substitute for Na. Axon diameter 834 $\mu$. Temperature 20° C.

previously that the Na efflux from unpoisoned *Sepia* fibres is increased by replacing external Na with choline or by replacing NaCl with dextrose (Hodgkin & Keynes, 1955). Squid fibres behave in the same way and replacement of Na with Li causes about the same increase as replacement with choline. In these respects the behaviour of cephalopod axons differs from frog muscle, in which replacement of Na with Li or choline normally causes a marked reduction of Na efflux (Keynes & Swan, 1959). The increase in efflux on removing Na is not understood, but seems inconsistent with there being much exchange diffusion in the normal fibre. However, in the poisoned or partially poisoned fibre, or in one which has been partially restored with ATP, complete removal of Na either has little effect or causes a drop in Na efflux. After an ATP injection the Na efflux into a K-free solution was reduced to 0·6 by replacing Na with Li. On the other hand, after an injection of arginine phosphate, at a time when the

Na efflux should have been K-sensitive, removal of external Na increased the Na efflux by about 10%.

Figure 5 provides further evidence that the initial effect of cyanide may be to increase exchange diffusion. The experiment was similar to that in Fig. 1 except that it was carried out in a lithium solution which was free from both Na and K. In contrast to Fig. 1, in which external Na was present, there was no transient rise on applying cyanide and the sodium efflux fell to the fully poisoned level without passing through a maximum.

In order to obtain more information about the possibility of exchange diffusion, the relation between external sodium concentration and Na efflux was investigated in an axon poisoned with 0·2 mM DNP at pH 8. Although the result was not inconsistent with there being some exchange diffusion it did not fit into any simple picture. In normal fibres removal of Na caused the Na efflux to increase along an S-shaped curve. After poisoning with 0·2 mM DNP at pH 8 reducing Na to half caused the Na efflux into a K-free solution to fall to 0·6. However, further reductions of Na caused a rise similar to that seen in normal fibres. Very little can be said about this result until the effect of 0·2 mM DNP at pH 8 on Na influx has been investigated.

## DISCUSSION

Although the analysis of the mechanism of ion transport is still incomplete, the experiments do seem to fit rather well with the idea that arginine phosphate is needed for the normal operation of the mechanism which extrudes Na and absorbs K in giant axons. If this conclusion is correct it raises two distinct questions. In the first place one would like to know whether arginine phosphate is directly concerned in handing on phosphate-bond energy to the membrane or whether, as seems more probable, it influences the transport mechanism by maintaining a high ATP:ADP ratio near the membrane. This point was discussed briefly in the previous paper and can reasonably be left until there is more information about the concentrations of ATP and ADP under different conditions. The other question which will be asked is whether the present set of experiments provide any clues as to the nature of the ionic transport mechanism in the membrane. In one model of cation transport (see Glynn, 1957), it is assumed that K and Na are carried through the membrane by the complexes KX and NaY. X and Y are carriers which can only move easily through the membrane when combined with ions. It is also assumed that metabolism converts X to Y on the 'inside' of the membrane and that the converse reaction takes place on the 'outside' of the membrane. Some of our results can be explained by supposing that the conversion of potassium carriers to sodium carriers on the 'inside' ($X \rightarrow Y$) requires ATP and that the converse reaction on the 'outside' is driven by arginine phosphate or by something derived from it (for example, a lipoid-soluble intermediate). In the normal fibre removal of external K blocks the out-

ward movement of Na because the carriers pile up in the potassium form (X) on the outside of the membrane and are unable to move inwards in the absence of external K. Mild inhibition of metabolism, which removes arginine phosphate but not ATP, stops the conversion of sodium carriers to potassium carriers on the 'outside' and allows a sodium exchange to proceed. This exchange is not affected by removing K from the external medium. More powerful inhibition, which removes ATP as well as arginine phosphate, stops sodium carriers being formed, with the result that the sodium efflux falls to a low value. On this view injection of ATP promotes mainly a sodium exchange, whereas injection of enough arginine phosphate to give ATP and a surplus of arginine phosphate enables both reactions to proceed and restores the normal coupling between Na exit and K entry. There are some obvious difficulties with the idea and it is not hard to suggest other schemes which fit the facts reasonably well. Thus there might be two competing mechanisms for extruding Na, one coupled with K influx and requiring a high ATP:ADP ratio, the other uncoupled and operating at a lower ATP:ADP ratio. Cation transport is still very far from being understood, but it seems possible that further investigation of the action of high energy phosphate compounds may throw light not only on the link with metabolism but also on events in the membrane itself.

SUMMARY

1. Application of 2 mM-CN, or 0·2 mM DNP at pH 6·7, to a squid giant axon caused a transient rise in the efflux of Na into a K-free solution, which probably coincided with the period when the axon contained ATP but not arginine phosphate.

2. Application of 0·2 mM DNP at pH 8, in which arginine phosphate breaks down but ATP is retained, caused a sustained rise in the efflux of Na into a K-free solution and little change in the efflux into 10 mM-K. Under these conditions an injection of arginine phosphate temporarily restored K sensitivity by reducing the Na efflux into a K-free solution.

3. 0·2 mM DNP at pH 8 reduced K influx to about half.

4. The general conclusion from these and earlier results is that the normal coupling between K influx and Na efflux depends on the presence of arginine phosphate.

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# PAPER ELECTROPHORESIS OF THYROXINE IN TRIS-MALEATE BUFFER

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When thyroxine, in the absence of serum, is submitted to electrophoresis on filter paper in barbiturate buffer at pH 8·6, all the thyroxine remains at the origin. If, however, the electrophoresis is carried out on filter paper that has been soaked in phenylalanine, or if phenylalanine is added to the paper with the thyroxine, the thyroxine moves towards the anode at a speed between that of the $\alpha_1$- and $\alpha_2$-globulins of human serum (Myant & Osorio, 1960). The term 'phenylalanine effect' was suggested for this action of phenylalanine, and for that of any other substance that exerts the same effect on the electrophoretic mobility of thyroxine on paper. The cause of the phenylalanine effect is unknown. We suggested, however, that it might be due to blocking of thyroxine-binding sites on the filter paper, which normally prevent thyroxine from moving towards the anode.

It has recently been shown (Ingbar, 1958) that when radioactive thyroxine, in the presence of human serum, is submitted to paper electrophoresis in tris-maleate buffer at pH 8·6, most of the activity is distributed between two peaks, one at the inter-alpha zone and the other in front of the albumin band (pre-albumin zone). Our observations with barbiturate buffer raised the question as to whether the phenylalanine effect also occurs in the presence of tris-maleate buffer and, if so, how far it could account for Ingbar's observations. We have therefore studied the effect of phenylalanine on the electrophoretic mobility of thyroxine in tris-maleate buffer at different hydrogen-ion concentrations with and without human serum. Observations were also made on the partition of thyroxine between the inter-alpha and pre-albumin zones at different concentrations of thyroxine. In an attempt to explain why thyroxine does not move to the pre-albumin zone in the presence of serum in barbiturate buffer, observations were made on the movement of thyroxine on paper electrophoresis in two dimensions.

## METHODS

Paper electrophoresis was carried out at room temperature by methods already described (Myant & Osorio, 1960). The tris (2-amino-2-hydroxymethylpropane-1:3 diol) buffer was made by mixing solution *A* (equal volumes of 0·4 M tris and 0·4 M maleic acid) and solution *B* (0·2 N-NaOH) in the following volumes (ml.), and diluting to 1 l. with water: pH 7·6, 300 of *A* + 333 of *B*; pH 8·6, 250 of *A* + 405 of *B*; pH 9·2, 233 of *A* + 425 of *B*. The pH was checked with a pH-meter at room temperature and, if necessary, brought to the exact value by addition of the acid or alkaline component. The borate buffers were prepared as already described (Myant, 1957), the volume of 0·2 N-NaOH being adjusted to give the required pH. The method used for proteolytic digestion of serum has been described elsewhere (Myant & Osorio, 1960). In the experiments on the effect of thyroxine concentration upon the distribution of thyroxine in serum, the amount of thyroxine carried at each of the three zones was estimated from the proportion of the total radioactivity at each peak, and the concentration of added thyroxine in the serum. The endogenous thyroxine already present in the serum was ignored in the calculations. The radioactive L-thyroxine, of specific activity 30 $\mu$c/$\mu$g, was obtained from Abbott Laboratories Pacific Ltd., Chicago. Radioactivity on the paper strips was measured with a continuously recording scanner. The positions of the radioactive spots after two-dimensional electrophoresis were found by making autoradiographs with Ilford X-ray film.

## RESULTS

### *Effect of pH on mobility of thyroxine in the presence of serum or phenylalanine*

In each experiment two strips of filter paper were submitted to electrophoresis in parallel. Radioactive thyroxine in human serum (1 $\mu$g/100 ml. serum) was put on one of the papers; the other was soaked in a solution of phenylalanine (1 mg/ml.) and received radioactive thyroxine dissolved in the buffer (1 $\mu$g/100 ml.).

At pH 8·6 the bands of protein, shown by staining the paper, were similar in position to those observed after electrophoresis in barbiturate buffer at pH 8·6. No staining was visible anywhere in front of the albumin band. On the paper to which thyroxine had been added in serum the radioactivity was distributed in three peaks, one in the inter-alpha zone, one in the albumin and one in the pre-albumin zone (Fig. 1*a*). At this concentration of added thyroxine the inter-alpha zone always carried the highest proportion of the total activity on the paper, the proportion at each of the three peaks averaging 56, 8 and 34% respectively. On the phenylalanine-treated paper with thyroxine alone all the activity moved as a single peak to a position corresponding to the inter-alpha zone (Fig. 1*b*).

When the pH of the buffer was changed from 8·6 to 7·6 or 9·2, the distribution of thyroxine was altered, both on the phenylalanine-treated paper in the absence of serum, and in the presence of serum. At pH 7·6, on the paper to which thyroxine had been added in human serum, 90% of the activity was present at the inter-alpha zone; the peak at the pre-albumin zone was either absent or only just detectable (Fig. 2*a*). On the

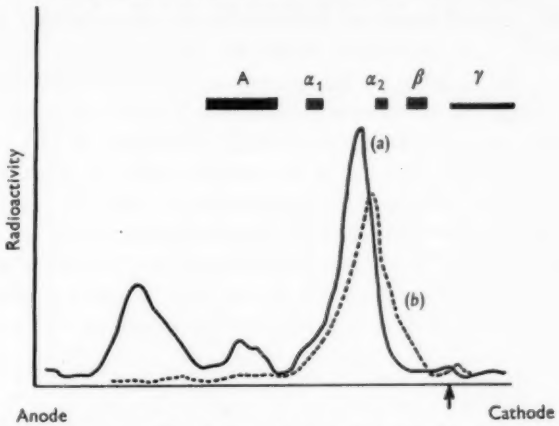


Fig. 1. Distribution of radioactivity along paper strip after electrophoresis in tris-maleate buffer at pH 8·6. *a*, radioactive thyroxine in human serum (1 $\mu$g/100 ml.) run on normal paper; *b*, radioactive thyroxine in buffer (1 $\mu$g/100 ml.) run on paper soaked in phenylalanine. The two strips of paper were run side by side in the same box. Arrow shows line of application of sample. Solid blocks show positions of protein bands (A = albumin). Note: the amount of radioactivity added to the paper in *a* was greater than in *b*.

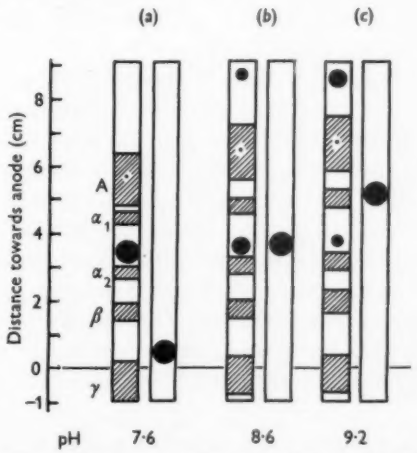


Fig. 2. Diagram to represent paper strips, showing effect of pH of tris-maleate buffer on distribution of thyroxine submitted to electrophoresis with serum on untreated paper and without serum on paper soaked in phenylalanine. The strip on the left of each pair was untreated and received radioactive thyroxine in serum (1 $\mu$g/100 ml.); that on the right was soaked in phenylalanine and received radioactive thyroxine in the buffer (1 $\mu$g/100 ml.). Shaded areas show positions of protein bands; solid circles show positions of peaks of radioactivity, the proportion of the total activity at each peak being shown roughly by the area of the circle. Vertical scale, distance towards anode from line of application of sample; horizontal scale, pH.

phenylalanine-treated paper all the activity remained within a few millimetres of the line of application of the sample.

At pH 9·2, the activity on the paper that had received the thyroxine in human serum was distributed in the same three peaks as at pH 8·6, but there was an increase in the amount of activity in the pre-albumin zone at the expense of that in the inter-alpha zone. At the concentration of added thyroxine used in these experiments (1 $\mu$g/100 ml.), an average of 40% of the total activity was in the inter-alpha zone, 9% was in the albumin band and 48% in the pre-albumin zone. On the phenylalanine-treated paper with thyroxine alone all the activity was present as a single peak at, or just in front of, a position corresponding to the $\alpha_1$-globulin (Fig. 2*c*).

### *Relation between concentration of thyroxine and its electrophoretic distribution in serum*

If the concentration of added thyroxine is increased from 1 to 500 $\mu$g/100 ml., there is a change in the partition of thyroxine between the inter-alpha, albumin and pre-albumin zones, the changes depending to some extent on the pH of the buffer. Figure 3 shows the amount of thyroxine carried in each of the three zones at different concentrations of thyroxine, as estimated from the distribution of radioactive thyroxine in serum submitted to paper electrophoresis.

When the pH of the buffer was 8·6, the inter-alpha zone acquired the highest proportion of the total thyroxine at the lowest concentrations. As the concentration was raised, however, the amount in the inter-alpha zone reached a maximum value corresponding to 20–30 $\mu$g/100 ml. serum, while that in the other two zones continued to increase to much higher values. When the total thyroxine concentration was 50 $\mu$g/100 ml., or higher, the pre-albumin zone always carried more than the inter-alpha zone. The amount in the pre-albumin zone rose to a maximum corresponding to 200–230 $\mu$g/100 ml. serum. The amount in the albumin zone increased slowly at first, but rose more steeply as the pre-albumin zone approached saturation, and eventually exceeded that in either of the other two zones.

At pH 9·2 the inter-alpha zone acquired a smaller proportion of the total thyroxine at low concentrations than was the case at pH 8·6. As the concentration was raised, however, the distribution of thyroxine at pH 9·2 became indistinguishable from that at pH 8·6. At both pH's the inter-alpha zone was saturated at a total thyroxine concentration of 20–30 $\mu$g/100 ml. and the pre-albumin zone at 200–230 $\mu$g/100 ml.

When the electrophoresis was carried out at pH 7·6, all the thyroxine was divided between albumin and the inter-alpha zone at all concentrations

Amount of thyroxine carried in inter-alpha, albumin and pre-albumin zones ($\mu$g/100 ml. serum)

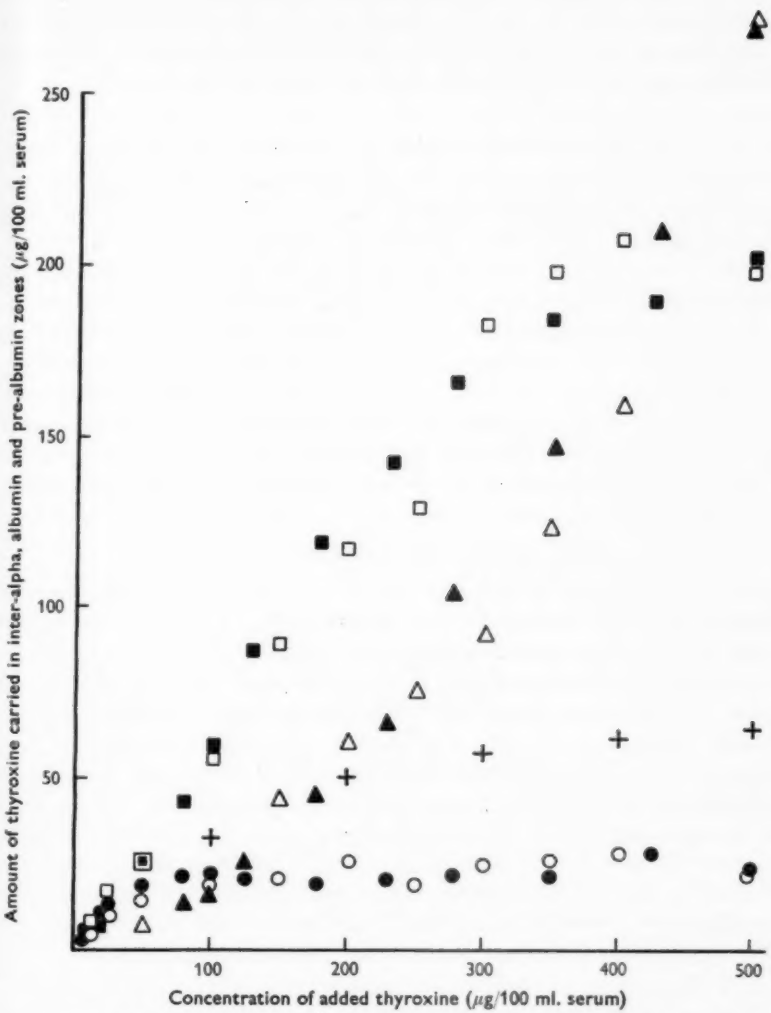


Fig. 3. Relation between concentration of added radioactive thyroxine in serum and amount of thyroxine carried at the inter-alpha (circles), albumin (triangles) and pre-albumin (squares) zones after electrophoresis in tris-maleate buffer at pH 9·2 (open symbols) and 8·6 (filled symbols). Crosses (+) show amount of thyroxine carried in inter-alpha zone in tris-maleate at pH 7·6. Each value averaged from several observations on different samples of serum from the same normal adult human being. The endogenous thyroxine in the serum (about 5 μg/100 ml.) has been ignored in all calculations.

from 1 to 500 $\mu$g/100 ml., none moving in the pre-albumin zone at any concentration tested. The amount carried in the inter-alpha zone increased throughout the whole of this range, reaching a value of about 60 $\mu$g/100 ml. when the concentration of added thyroxine was 500 $\mu$g/100 ml.

*Electrophoretic distribution of endogenous thyroxine*

A single observation was made on the distribution of endogenous thyroxine in the serum of a thyrotoxic patient. A sample of blood was taken from the patient 4 days after she had been given a dose of 7 mc of $^{131}$I, and the serum was submitted to paper electrophoresis in tris-maleate buffer at pH 8·6. The distribution of radioactivity was similar to that in serum to which radioactive thyroxine had been added at a concentration of 1 $\mu$g/100 ml., the proportions of the total activity at the inter-alpha, albumin and pre-albumin zones averaging 57, 6 and 35% respectively. It was possible to demonstrate that nearly all the activity on the paper was due to radioactive thyroxine, by showing that 90% of the activity at each of the three peaks was removed by washing the paper with ethanol.

*Electrophoretic distribution of thyroxine in borate buffer*

Since the electrophoretic behaviour of thyroxine in tris-maleate buffer differs so markedly from that in barbiturate buffer, it was of some interest to see how thyroxine behaves in the presence of another buffer. Accordingly, all the experiments described up to this point were repeated with borate buffer. All the results obtained were similar to those obtained with tris-maleate buffer.

The distribution of thyroxine in the presence of serum, and when tested by itself on phenylalanine-treated paper, was the same in borate as in tris-maleate buffer at pH 7·6, 8·6 and 9·2; so, also, was the effect of raising the concentration of thyroxine on the partition of thyroxine between the inter-alpha, albumin and pre-albumin zones. Finally, the distribution of endogenous thyroxine in serum tested in borate buffer at pH 8·6 did not differ significantly from that observed in tris-maleate at pH 8·6.

*Movement of thyroxine to the pre-albumin zone during electrophoresis in two dimensions*

The failure of any thyroxine to move ahead of the albumin in tris-maleate at pH 7·6 or in barbiturate at pH 8·6 calls for some explanation. One possibility is that under these conditions the binding substance, presumed to be responsible for carrying thyroxine to the pre-albumin zone, moves ahead of the albumin, but is unable to bind thyroxine. In order to test this possibility paper electrophoresis of thyroxine in serum

was carried out in two dimensions, first in barbiturate, and then in tris-maleate at pH 8·6.

A sample of serum, without any added thyroxine, was put on to a strip of filter paper and submitted to electrophoresis in barbiturate buffer at pH 8·6. The paper was then removed and cut longitudinally into two pieces. One piece was dried and stained to show the positions of the protein bands. The other was then gently pressed down along the cathodal edge of a square sheet of filter paper prepared for electrophoresis in tris-maleate buffer at pH 8·6, and already in position in the box. About 50 $\mu$l. of a solution of radioactive thyroxine in tris-maleate buffer at pH 8·6 was put on the square sheet of paper along a line 1 cm in front of the edge of the strip of paper used in the first dimension, and extending along the whole of its length. Electrophoresis was then carried out for 18 hr. The distribution of radioactivity, as shown on an autoradiograph made from the dried paper (Fig. 4), showed that the thyroxine placed opposite the pre-albumin zone in the first dimension was carried towards the anode in the second dimension. The same result as that shown in Fig. 4 was obtained when tris-maleate at pH 7·6 was used in the first dimension, in place of barbiturate at pH 8·6.

Movement of thyroxine at the pre-albumin zone could be demonstrated in the second dimension only if the experiment was done in the way described above. If the radioactive thyroxine was put on the strip of paper used in the first dimension, instead of 1 cm in front of it on the second paper, none of the activity in the pre-albumin zone moved towards the anode during electrophoresis in tris-maleate buffer at pH 8·6. Presumably the buffer in the paper strip used for the first dimension prevented the binding substance in the pre-albumin zone from binding thyroxine; binding could only occur in conditions in which the binding substance first came into contact with the thyroxine in the presence of the tris-maleate buffer used for the second dimension.

### *Effect of proteolytic enzymes on the electrophoretic distribution of thyroxine in serum*

In an earlier paper (Myant & Osorio, 1960) it was shown that when thyroxine is submitted to paper electrophoresis in the presence of a proteolytic digest of serum in barbiturate buffer, at pH 8·6 all the thyroxine moves to the inter-alpha zone, whereas at pH 7·6 it remains at the line of application. These experiments were repeated, with tris-maleate instead of barbiturate buffer. For each experiment two papers were run in parallel, one with radioactive thyroxine dissolved in normal serum (1 $\mu$g/100 ml.), and the other with radioactive thyroxine dissolved at the same concentration in serum that had been digested with papain.

At pH 8·6 the radioactivity in the proteolytic digest moved as a single peak to a position corresponding to the inter-alpha zone, there being no detectable activity in the pre-albumin zone. At pH 7·6 all the radioactive thyroxine added to the paper in the digested serum remained at the line of application of the sample; most of the radioactivity on the paper with normal serum moved as a single peak to the inter-alpha zone, as has already been shown in Fig. 2*a*.

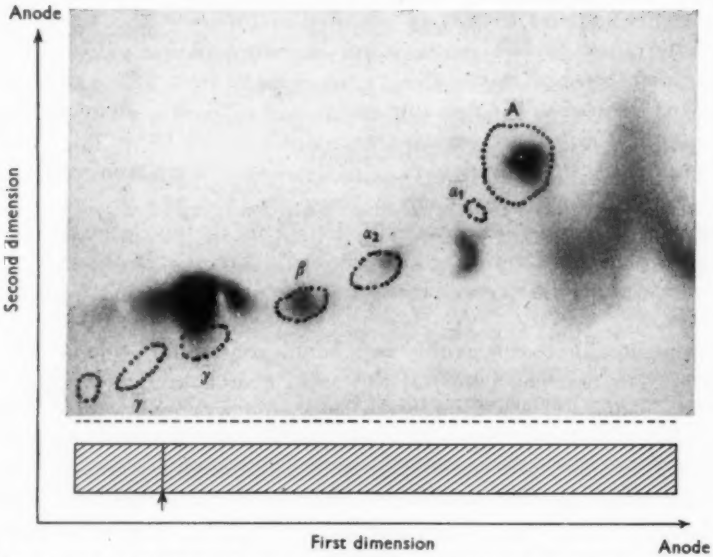


Fig. 4. Autoradiograph of two-dimensional paper electrophoresis of radioactive thyroxine in serum (10 μg/100 ml.). First dimension in barbiturate buffer at pH 8·6; second dimension in tris-maleate buffer at pH 8·6. Shaded area shows position of strip used for first dimension (line of application of serum shown by arrow), after being placed on square sheet of paper at start of run in second dimension. Radioactive thyroxine solution (50 μl.) was applied approximately along the horizontal dotted line for run in second dimension. Areas enclosed by dotted lines show positions of protein spots at end of run in second dimension, letters indicating serum protein fractions (A = albumin). Black spots show positions of radioactivity. Movement of radioactivity in pre-albumin zone shown by broad arrow-shaped area of blackening to right of albumin spot.

## DISCUSSION

The observations described in this paper show that the phenylalanine effect occurs in tris-maleate buffer, as well as in barbiturate. It cannot, however, account for the electrophoretic mobility of thyroxine in human serum, as observed in tris-maleate. In the first place, phenylalanine does not cause thyroxine by itself to move to the pre-albumin zone at pH 8·6

or 9·2, although at both pH's some thyroxine moves to the pre-albumin zone in the presence of serum (Fig. 2). Secondly, if thyroxine is submitted to paper electrophoresis on phenylalanine-treated paper at pH 7·6, it remains at or near the origin, whereas at this pH it moves in the inter-alpha zone in the presence of human serum. Our observations are, therefore, consistent with the view, now generally held, that when thyroxine moves towards the anode on paper electrophoresis in the presence of serum, it does so because it is bound to a specific substance in the serum whose electrophoretic mobility determines that of the thyroxine. Although there is no proof for this hypothesis, no other satisfactory explanation has been put forward to account for the fact that there is a limit to the amount of thyroxine that can move in the inter-alpha and pre-albumin zones, and for the purpose of this Discussion we shall assume the hypothesis to be true.

The electrophoretic distribution of thyroxine in serum in tris-maleate at pH 8·6 and 9·2 raises the possibility, first suggested by Ingbar (1958), that there are two specific thyroxine-binding substances in human serum. It has been suggested (Tata, 1960) that there is only one binding substance —the one responsible for carrying thyroxine to the inter-alpha zone in barbiturate—and that in the presence of tris-maleate at pH 8·6 this splits into two fractions, one remaining in the inter-alpha zone and the other moving to the pre-albumin zone. A similar explanation might also be put forward to account for the difference between the behaviour of thyroxine in tris-maleate at pH 8·6 and at pH 7·6 (Fig. 2). The binding capacity at the inter-alpha zone is higher in barbiturate than in tris-maleate at pH 8·6 (compare Robbins (1956) with Fig. 3), and this certainly suggests that the buffer influences the relative amounts of binding substance in the two zones. However, this cannot be the whole explanation of the failure of thyroxine to move in the pre-albumin zone in barbiturate, since the inter-alpha zone in barbiturate always carries less thyroxine than is carried in the two zones together in tris-maleate at pH 8·6. A comparison between the results of Robbins (1956) and those of Fig. 3 shows, for instance, that when the total thyroxine concentration is 100 $\mu$g/100 ml., in barbiturate the inter-alpha zone carries 35 $\mu$g/100 ml. whereas in tris-maleate at pH 8·6 the inter-alpha and pre-albumin zones together carry more than 80 $\mu$g/ml.

Electrophoresis in two dimensions shows that a binding substance does move to the pre-albumin zone in barbiturate and in tris-maleate at pH 7·6, although it is unable to adsorb thyroxine in these conditions. This is best explained by supposing that there are, in fact, two binding substances in human serum with different electrophoretic mobilities, one of which moves to the pre-albumin zone (pre-albumin binding substance) and is prevented

from binding thyroxine under certain conditions. The existence of two different binding substances would also explain why extracting serum with alcohol suppresses thyroxine-binding in the inter-alpha zone, but not in the pre-albumin zone (Osorio & Myant, unpublished observations). This hypothesis does not explain the high binding capacity at the inter-alpha zone in barbiturate and in tris-maleate at pH 7·6. As was suggested by Robbins (1956), part of the albumin fraction may, in some conditions, move in the inter-alpha zone. Alternatively, the binding capacity of the inter-alpha substance itself may be increased in barbiturate and in tris-maleate at pH 7·6.

When serum has been digested with proteolytic enzymes it no longer enables thyroxine to move in the pre-albumin zone during paper electrophoresis in tris-maleate at pH 8·6. This suggests that the pre-albumin binding substance is a protein. If so, its concentration in the serum must be very low, since protein at a concentration of 6 mg/100 ml. was detectable with the dye used for staining the paper, yet no staining was ever seen in front of the albumin band.

We have drawn attention elsewhere (Myant & Osorio, 1960) to the difficulty of correlating observations on the electrophoretic distribution of thyroxine in serum, if the observations are made by different methods. Rich & Bearn (1958) have shown that when thyroxine is submitted to electrophoresis in starch gel in the presence of serum, it moves in a zone ahead of the albumin. However, the substance responsible for this effect cannot be assumed to be the pre-albumin binding substance observed on paper electrophoresis in tris-maleate. On the contrary, Tata (1960) has suggested that the binding substance observed by Rich & Bearn is the same as that which carries thyroxine to the inter-alpha zone during paper electrophoresis.

It has generally been assumed that the substance that carries thyroxine to the inter-alpha zone on paper electrophoresis in barbiturate at pH 8·6 also binds most of the thyroxine circulating in the blood under physiological conditions (see, for instance, Robbins & Rall, 1957), though the evidence for this has always been indirect. Since it now appears that there are two specific binding substances in serum, this assumption must be re-examined. At pH 8·6 and 9·2 the pre-albumin substance has a binding capacity of more than 200 $\mu$g of thyroxine/100 ml. of serum, as shown by the maximum value approached at the highest concentrations tested (Fig. 3). This is about ten times the capacity of the inter-alpha substance. The binding affinity of the inter-alpha substance, however, must be at least ten times that of the pre-albumin substance. This is shown by the fact that with endogenous thyroxine, and at the lowest concentrations of added thyroxine, when the amount bound by each substance is

proportional to the product of its affinity and its capacity (Myant & Osorio, 1959), the thyroxine is shared about equally between the two substances.

Unfortunately, the conditions necessary for adequate separation of the serum proteins by paper electrophoresis are not physiological; nor is it easy to deduce how thyroxine is distributed between the different protein fractions *in vivo*, by extrapolation from observations *in vitro*. As the pH of the tris-maleate buffer is reduced from 9·2 to 8·6, there is a slight increase in the proportion of the thyroxine carried in the inter-alpha zone at low concentrations, but no change in the binding capacity at either the inter-alpha or the pre-albumin zone (Fig. 3). This suggests that a change in the pH towards the physiological level increases the binding affinity of the inter-alpha substance relative to that of the pre-albumin. The findings with tris-maleate at pH 7·6 are consistent with this, although some other effect, as discussed above, must be assumed in order to account for the high capacity at the inter-alpha zone at this pH.

The effect of pH might suggest that the pre-albumin substance plays little part in the transport of thyroid hormone *in vivo* and that the distribution of thyroxine observed by paper electrophoresis in barbiturate buffer is closer to the physiological than that observed in tris-maleate at pH 8·6. On the other hand, it may be relevant that at pH 8·6 the pre-albumin substance binds nearly half the thyroxine in serum in three different buffers—ammonium carbonate (Beierwaltes & Robbins, 1959), tris-maleate and borate. Until something is known about the mechanism by which the pH and composition of the buffer influence the behaviour of thyroxine-binding substances in serum, it would be unwise to draw any conclusions as to which results most closely reflect the distribution of thyroxine *in vivo*.

## SUMMARY

1. In tris-maleate buffer the electrophoretic mobility of thyroxine alone on phenylalanine-treated paper differs from that of thyroxine in human serum, at pH 8·6 and at pH 7·6. It is concluded that the distribution of thyroxine in serum, as observed by paper electrophoresis, is not due to the serum exerting an action analogous to that of phenylalanine. It is more likely that it is determined by the presence of thyroxine-binding substances in the serum.

2. During paper electrophoresis in barbiturate or in tris-maleate at pH 7·6 no thyroxine moves ahead of the albumin, although in both conditions a binding substance may be demonstrated in the pre-albumin zone by electrophoresis in a second dimension.

3. If the pH of tris-maleate buffer is reduced from 9·2 to 8·6, there is

an increase in the binding-affinity of the inter-alpha substance relative to that of the binding substance moving in the pre-albumin zone. The binding of thyroxine by the serum proteins in physiological conditions is discussed in relation to this finding.

4. When thyroxine is submitted to paper electrophoresis in the presence of papain-treated serum, it no longer moves in the pre-albumin zone in tris-maleate at pH 8·6. This is consistent with the view that the binding substance responsible for carrying thyroxine in the pre-albumin zone is a protein.

We are indebted to Dr D. J. R. Laurence for discussion. One of us (C.O.) was in receipt of a Research Fellowship from The Wellcome Trust.

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PROCEEDINGS OF
THE PHYSIOLOGICAL SOCIETY

UNIVERSITY COLLEGE LONDON MEETING

25–26 *March* 1960

**A model heart with independent control of strength and frequency of beat and duration of systole.** By L. E. Bayliss. *Department of Physiology, University College London*

**A low-capacitance, low-output impedance, stimulus coupler.** By G. D. Dawson, J. R. Pitman and D. R. Wilkie. *Department of Physiology, University College London, and the M.R.C. Neurological Research Unit, The National Hospital, Queen Square, London*

The stimulus output unit to be demonstrated is controlled by light, which allows its capacity to earth to be reduced to the minimum set by its dimensions. Also, since none of the power in the stimulus is transmitted through the controlling link, it can have a low output impedance (in this unit, less than 1250 Ω during the pulse) and considerable power can be delivered to the tissue being stimulated, e.g. when stimulating a nerve through the skin in man. In this respect it may have a considerable advantage over the radio-frequency links previously described (see Donaldson, 1958). Including the batteries to provide the output power, the unit occupies a cube of about 5 cm side and has a 'free space' capacity of 8·5 pF, including output leads 30 cm long. If it is surrounded by a cubical earthed screen with a side of 10 cm, the capacity to earth is increased to 16·5 pF. The amplitude of the stimulating pulse is controlled in the output unit, and it has a maximum of 60 V with a rise time of 5 μsec. The duration of the stimulus is controlled by the duration of the light pulse shone on the output unit.

The light pulse to trigger the unit is obtained from a small tubular type 60 V neon lamp (Radio Spares). This is driven by a pulse generator so that during a stimulus a maximum current of 5 mA is passed through it, while between stimuli the current is maintained at a minimum of 0·25 mA, to avoid delay in striking. The light from the neon lamp is conducted along a Perspex light guide 10 cm long, to a germanium photocell in the output unit. High amplification after the photocell makes small variations of the light output of the neon relatively unimportant; a light varying

sinusoidally will switch the output transistor fully on and off and give a rectangular output. The light guide also excludes light from other sources which would otherwise trigger the stimulator. To prevent the cut-off current through the output transistor (20 $\mu$A) affecting the preparation, the output is resistance-capacity coupled. The capacitor is sufficiently large to pass a 1 msec rectangular pulse to a 1 k$\Omega$ load with a sag of not more than 10 %. If the minimum current in the output is not important this RC coupling may be omitted and the output pulse will then remain rectangular for a period limited only by the capacity of the battery B2.

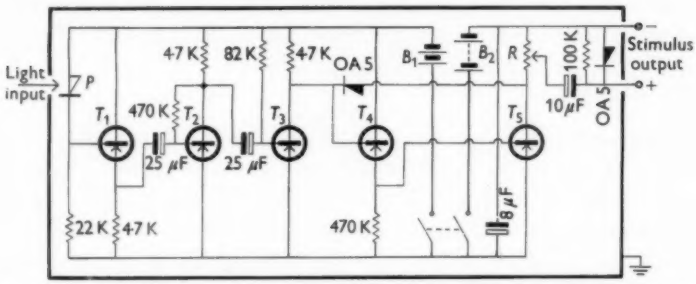


Fig. 1. Circuit of light-controlled stimulator. The input photocell $P$ is a Standard Telephones and Cables type P50A. Amplifying transistors $T_1$ to $T_4$, Newmarket type V6/4R. Output transistor $T_5$ is a silicon p-n-p type with a low cut-off current, Mullard OC 203. $R$, the output voltage control, is a wire-wound potentiometer of 5 k$\Omega$. The amplifier power supply, $B_1$, is two Mallory cells, type RM625R, in series giving 2·7 V. The output power supply, $B_2$, consists of two 22·5 V batteries, Batrymax type B122, and one 15 V battery, Batrymax type B121, in series.

The diode from the collector of the output transistor $T_5$ to the base of $T_4$ limits the current which $T_4$ can pass into the base of $T_5$ under conditions in which the potential difference between the emitter and collector of $T_5$ is very low. Such a heavy current in the base circuit of $T_5$ could damage it and in addition causes serious carrier storage effects. These slow down greatly the rate at which the output transistor can be switched off at the end of the trigger pulse.

The capacity to earth of this output unit is some 10 times less than that of a good quality pulse transformer designed to pass a rectangular pulse lasting 1 msec. This should simplify an assessment of the part played in the production of stimulus artifacts by capacitative leakage. If only small stimulating currents are required, from a high impedance source, a vacuum photocell and battery may be used without further complication.

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**The action of ether on the discharges of spindle motor fibres.** By K. Diete-Spiff and J. E. Pascoe. *Department of Physiology, University College London*

**Bisection of isolated glomeruli.** By W. F. Cook. *Department of Biophysics, University College London*

**A theory to account for the absence of boutons in silver preparations of the cerebral cortex, based on a study of axon terminals by light and electron microscopy.** By B. B. Boycott, E. G. Gray and R. W. Guillery. *Departments of Zoology and Anatomy, University College London*

One of the great obstacles to the study of synaptic connexions in the neocortex has been the virtual absence of classical boutons in silver preparations. The classical rings and bulbs of the spinal cord and brain stem cannot be observed in the cortex, so that there the boutons have been considered too small to be resolved by light microscopy, or their presence has been denied.

On the other hand, in the lizard brain large argyrophilic rings are conspicuous in the hippocampus and dorsal cochlear grey. These rings vary from 2 to 4$\mu$ in diameter and are closely associated with dense clumps of mitochondria. The same regions have also been examined by electron microscopy with osmium fixation, alcoholic phosphotungstic acid staining and araldite embedding. For comparison the visual cortex of the rat has been examined by the same method (see Gray, 1959).

The electron micrographs of the lizard's brain show large endings, up to 4·5$\mu$ in diameter, containing numerous mitochondria and synaptic vesicles. Occasionally these endings (Fig. 1) show a bundle of neurofilaments (each filament 50–100 Å thick) lying in the form of a ring (2–4$\mu$ in diameter). These rings appear sectioned in all planes and, when sectioned radially, two groups of dots appear at opposite poles of the profile of the ending. Each ring is composed of 10–50 or more filaments. Sometimes, when the non-myelinated axon is seen continuous with the ending, a tail of filaments extends into the axon from the ring. The post-synaptic component with the thickened contact regions sometimes appears in the plane of section. Size, shape, location and the relation to mitochondria suggest that these rings correspond with those seen under the light microscope after silver staining. Neurofilaments impregnated or encrusted with silver have been thought to correspond to the neurofibrils of light microscopy (see Palay & Palade, 1955).

Electron micrographs of the neocortex show numerous endings, and since the majority exceed 0·5$\mu$ in diameter they are within the limits of

resolution of the light microscope. These cortical boutons contain many synaptic vesicles and mitochondria, but no neurofilaments (either in the form of rings or otherwise). Even in the pre-terminal non-myelinated axons neurofilaments are uncommon.

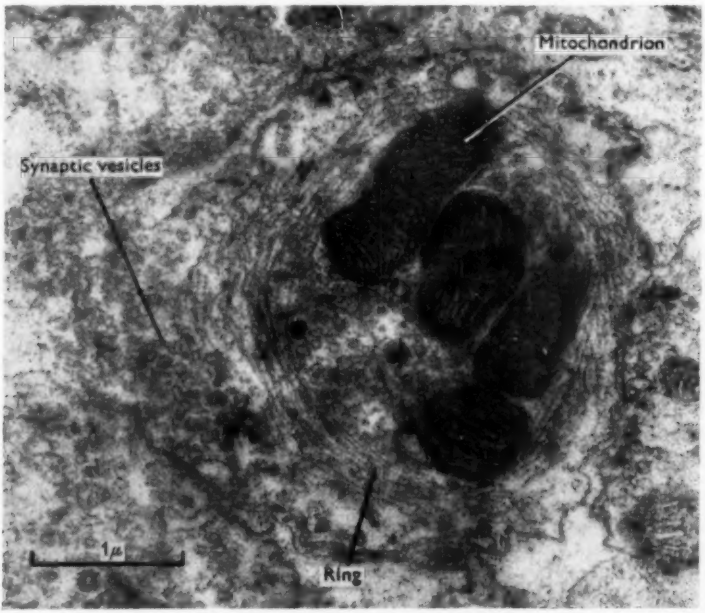


Fig. 1. A nerve ending of the dorsal cochlear grey of the lizard. The thickening of the synaptic membranes does not appear in the plane of section.

Work in progress with light and electron microscopy on boutons of other regions has shown similar rings in the rat ventral horn. On the present interpretation, that the neurofilaments of electron microscopy form the basis for the argyrophilic classical boutons, one would expect to find neurofilaments wherever there are boutons. Further, it should prove possible to correlate the distribution of the filaments with the morphology of the boutons. This would support the theory that the neurofilaments of electron microscopy correspond with the argyrophilic neurofibrillae of light microscopy. It would also suggest that since the endings of the neocortex contain no neurofilaments they are never likely to be detected by neurofibrillar techniques. Further, it appears probable that rings are valid criteria for nerve endings, but since nerve endings do not always contain neurofilaments the neurofibrillar techniques are seldom likely to reveal

the total content of endings in a given region of the nervous system (see also Armstrong, Richardson & Young, 1956).

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**A method for locating the cholinesterase of a mammalian myoneural junction by electron microscopy.** By R. I. Birks* and Lucy M. Brown. *Departments of Biophysics and Physiology, University College London*

Histochemical studies of cholinesterase activity at myoneural junctions have shown that the enzyme is probably located in narrow bands about 1 $\mu$ apart on the muscle surface beneath the nerve ending (Couteaux & Taxi, 1952). Lehrer & Ornstein (1959), using a diazo-coupling method for the electron microscope, have confirmed the suggestion that the enzyme is located in the infoldings of the post-synaptic membrane.

Fig. 1. Whole mount of myoneural junction incubated in the silver medium at pH 6·5 for 30 min.

We have used a modification of Koelle's method (Koelle & Friedenwald, 1949). Guinea-pig serratus anterior was fixed for 30 min at 0° C in 1% buffered osmium tetroxide. Small blocks containing myoneural junctions were dissected from the thin borders of the muscle and incubated for 5–60 min at 40° C in a medium containing 20 mM acetate buffer and 4 mM acetyl thiocholine. In the Koelle medium 4 mM-$CuSO_4$ and 20 mM

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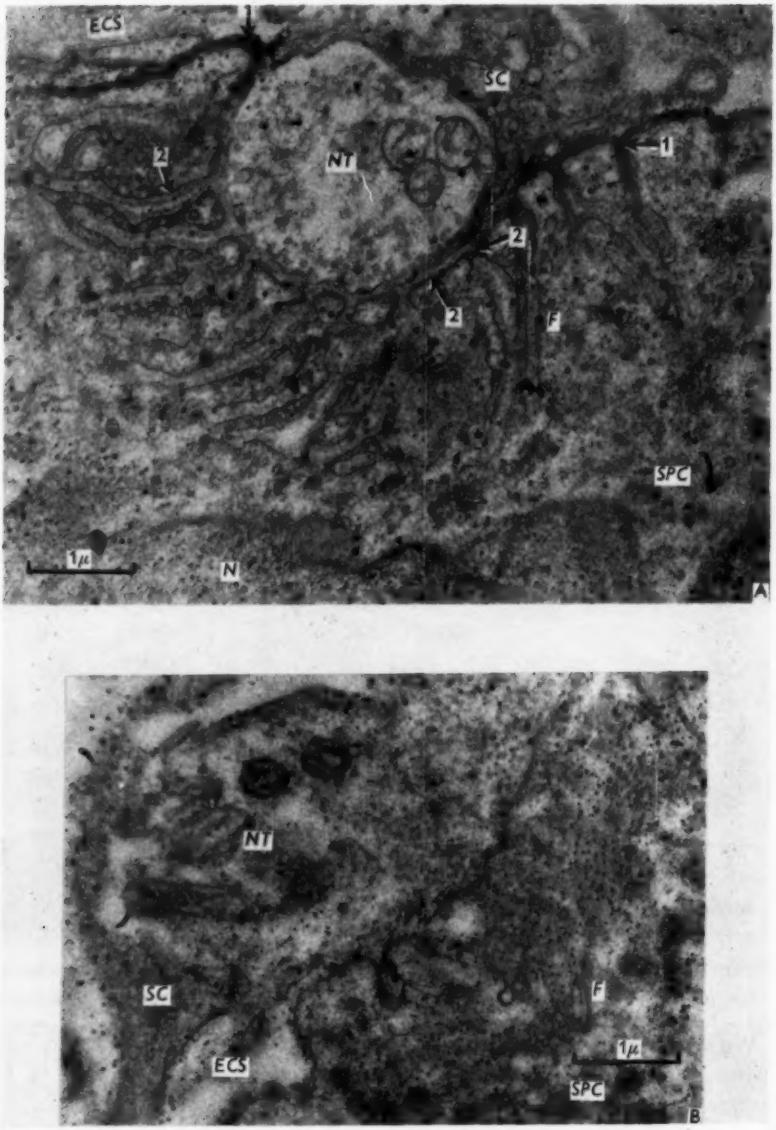


Fig. 2. Low-power electron micrographs of 0·1 $\mu$ sections. A. Incubation as in Fig. 1; 1: extra-synaptic site; 2: synaptic deposition of stain. B. Incubation with addition of $10^{-5}$ M eserine. *ECS*, Extracellular space; *F*, post-synaptic fold; *NT*, nerve terminal; *N*, nucleus; *SC*, Schwann cell; *SPC*, sole plate cytoplasm.

glycine provided copper for combination with the enzymically liberated thiocholine. The blocks were dehydrated and embedded in araldite.

When tissues incubated in Koelle's medium at pH 6 were examined in the electron microscope, needle-shaped crystals of copper thiocholine sulphate up to 1 $\mu$ in length could be seen in the junctional area. There was considerable local damage, probably caused by thiocholine, since it did not occur when $10^{-5}$ M eserine was present. The damage was diminished at pH 4·5 or when glycine was omitted and the copper concentration reduced to 0·5 mM, but it was sufficient to prevent accurate localization.

Substitution of 0·5 mM silver sulphate for the copper and glycine eliminated the local damage and led to the deposition of a black amorphous precipitate in well-defined areas of the junction (Figs. 1 and 2A). The localization was independent of pH in the range 4·5–6·5 and no selective staining occurred in the presence of eserine, although a granular precipitate was randomly distributed throughout the tissue (Fig. 2B).

Two sites of selective staining were observed. There was a dense deposit at a site 1–2 $\mu$ wide on the outer diffuse layer of the muscle membrane complex (cf. Robertson, 1960) bordering the junctional region and extending into the superficial parts of the folds not covered by nerve ending. In the region under the nerve terminal some areas of the unit membrane, but not of the outer diffuse layer, showed heightened contrast. The stain appeared to be confined mainly to the superficial parts of the folded postsynaptic membrane. The sites of dense staining are thought to indicate membrane areas of intense cholinesterase activity. Other areas of slight staining occurred, but it was not possible to define these sites accurately.

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**A simple method of indicating the phase of respiration on the Y2 beam of the Cossor oscilloscope (1049).** By G. READ. *Department of Physiology, University College London*

**Granulation tissue resorption and wound contraction.** By D. W. JAMES and J. F. NEWCOMBE. *Department of Anatomy, University College London*

The scar resulting from the healing of a large excised skin wound is considerably smaller in area than was the original wound, since in the early stages of repair the intact skin surrounding the deficit is drawn

inwards by the contraction of the granulation tissue within the wound (e.g. Abercrombie, Flint & James, 1956). This process is known as wound contraction. The present experiments were designed to investigate changes in the composition of granulation tissue associated with the wound contraction it mediates.

Twenty rabbits were wounded bilaterally by excision of $2 \times 2$ cm squares of thoracic wall skin. Wounds were splinted with Perspex frames glued to the surrounding skin to impede contraction. On the 10th post-operative day $1 \times 1$ cm squares were tattooed on the granulation tissue. These areas were excised for analysis on the 10th or 12th day with splints *in situ*, or on the 12th day when rapid and extensive contraction had been induced by splint removal on the 10th.

No absolute differences in wet weight, collagen, or non-collagen nitrogen contents of equivalent areas from splinted wounds at 10 and 12 days were detected, but such areas from wounds contracting freely between the 10th and 12th days weighed less, and contained less collagen and non-collagen nitrogen. Contraction was accompanied by increase per unit area in the parameters measured, but no differences were detected between them in the three treatment groups when expressed in terms of unit wet weight.

Thus absolute resorption of material, including collagen, occurred between 10 and 12 days only if free contraction was permitted, but there were no detectable changes in tissue composition (as judged by the indices measured) associated with this resorption. Quantitative histological measurements of comparable material again suggest stability of composition, since contraction does not apparently affect the number of cells per unit volume, although it is accompanied by an absolute reduction in cell population.

The observed material loss is a consequence of contraction and not its cause (Abercrombie, James & Newcombe, 1960). Simple material withdrawal would not explain the increased weight per unit area (i.e. thickness) associated with the area reduction of contraction.

It seems likely from these experiments that the changing morphology of granulation tissue during contraction triggers its own catabolism.

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**A method for mixing fluids within a microcalorimeter.** By E. W. Clarke. *Department of Physiology, The Queen's University of Belfast*

The measurement of the heat resulting from the mixing of two solutions must take into account the heat produced mechanically by the mixing

process. To find a way of keeping this mechanical heat both small and reproducible became the main problem in some recent experiments to measure the heat of mixing of various dilute isotonic solutions. The method to be described was found to be by far the most satisfactory of several that were tried.

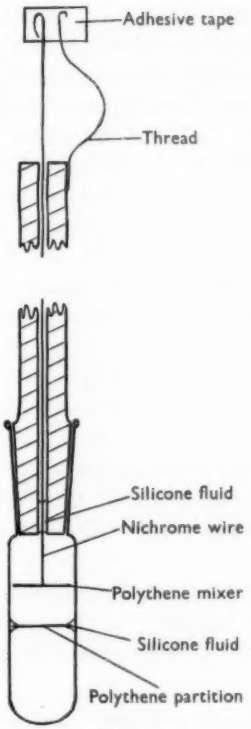


Fig. 1

The reaction cell used in the microcalorimeter (see Clarke & Rothschild, 1957) is represented in Fig. 1. It consists of a glass tube of approximately 2·5 ml. capacity having a ground glass stopper. The stopper and its long stem (25 cm) are made from a 2 mm bore capillary tubing. The inner surface of the reaction cell is rendered water-repellent by treatment with a silicone. After placing one of the test solutions in the cell its surface is covered by a disk of polythene film cut slightly smaller than the internal diameter of the cell. The fluid surface remaining exposed between the disk and walls of the cell is then sealed with a little silicone fluid (viscosity 1000 centistokes). The second solution is carefully added above the partition so formed and the stopper inserted. Another polythene disk is used

to break the seal and mix the cell contents. It is supported on a piece of 0·2 mm diameter nichrome wire which passes up the capillary. When the seal has been broken the two disks remain together during the two or three vertical movements required to bring about complete mixing. For precise measurements a stop is used to prevent the stirrer striking the top or bottom of the cell, and may be made by placing some cellulose adhesive tape round the upper end of the wire and joining this to the top of the stem with an appropriate length of sewing thread. With this arrangement the total amount of heat produced by breaking the seal and mixing the contents is in the order of 0·2 millicalories. The volume of the upper solution may be accurately defined by sealing the bottom end of the capillary with some low-viscosity silicone fluid introduced through a fine tube. The volume of the first solution and the partition are found from their densities and weights in the cell. The volume of the cell is measured with the capillary sealed, and the above volumes, plus the small calculated volume of the mixer, are subtracted from it to give the volume of the upper solution.

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**Acetylcholine, 5-hydroxytryptamine and a new kinin in hornet venom.** By K. D. BHOOLA, J. D. CALLE and M. SCHACHTER. *Department of Physiology, University College London*

**Effect of serum kallikrein and bradykinin on capillary permeability.** By K. D. BHOOLA, J. D. CALLE and M. SCHACHTER. *Department of Physiology, University College London*

**Changes in excitability of primary receptor units in the pad of the cat with varying patterns of mechanical stimulation.** By CHRISTINE J. ARMETT, J. A. B. GRAY and R. HUNSPERGER. *Department of Physiology, University College London*

**Methods for the examination of nucleoprotein structures with the electron microscope.** By H. E. HUXLEY and G. ZUBAY. *Department of Biophysics, University College London, and M.R.C. Biophysics Research Unit, King's College, London, W.C. 2 and Department of Biophysics, University College London*

During the last few years, electron microscopy has been applied very successfully in the study of a large number of biological structures—nerves, muscles, mitochondria, secretory cells, motor end-plates, for

example—but the results have been somewhat disappointing in the case of structures in which nucleic acids play a major part—chromosomes, viruses and microsomal particles. It appeared likely that conventional methods of fixation and staining, originally developed for structures consisting largely of protein, might require modification in these other cases.

With this end in mind, we have used oriented samples of nucleohistone from calf thymus as a test substance. We have examined thin sections of embedded material fixed and stained in different ways to try to find out which procedures gave a product in which the orientation was preserved and in which fine structure, compatible with the known physico-chemical and X-ray diffraction data, was visible.

We found that provided calcium was present in the buffered osmic acid fixative, and that araldite was used as the embedding medium, very satisfactory preservation of orientation and fine structure was obtained. We further found that uranyl acetate appeared to act as an intense and strongly preferential stain for nucleic acids, a result we were able to confirm by direct chemical measurements and by a comparison of adjacent sections of whole thymus tissue, stained with uranyl acetate for the electron microscope, and by the Feulgen procedure for the light microscope.

The demonstration illustrates these results and also shows the appearance of microsomal particles and some of the small viruses stained in the same way. The latter cases are also compared with results obtained by the negative staining method (in which the outline of the particle as a whole is shown up); there is strong evidence for the existence of a protein shell in the case of the viruses, but not in the case of the microsomal particles.

**Simultaneous recording of activity and rate of chirping in the young chick.** By ELEANOR ZAIMIS. *Department of Pharmacology, Royal Free Hospital School of Medicine, London, W.C.* 1

Young chicks (3–10 days old) have been found very useful for the study of the central actions of various drugs. For example, from the responses obtained one can easily differentiate between lysergic acid diethylamide and mescaline, between the amphetamines and other central nervous-system stimulant drugs, between sympathomimetic amines producing central inhibition and those producing central stimulation. Of the various responses which such drugs produce alterations in the rate of chirping and in general motor activity can be recorded in the following manner.

The chick is placed in a wooden box covered with a sliding Perspex lid and lined with acoustic tiles which reduce the pick-up of external noise. Inside the box there are (*a*) a crystal microphone built into a recess of one wall, and (*b*) a false floor fixed at its centre to the diaphragm of a modified telephone earpiece. The movements of the false floor are damped by strips of rubber bands stretched between it and the floor of the box. The output from the microphone is amplified, filtered and shaped, and the shaped pulses are passed into a low-frequency rate-meter (Kitchen, 1959). The output of the rate-meter and that of the floor transducer are fed directly on to the galvanometers of an ultra-violet recorder (Type 1050, New Electronic Products). The voltage output of the rate-meter is proportionate to the frequency of the chirping of the chick and that of the earpiece gives an approximate indication of its activity. Both outputs are recorded simultaneously on paper.

The apparatus was designed and built by Mr P. Inglis-Smith and the purchase of the Ultra-Violet Recorder was greatly assisted by a grant from the National Research Development Corporation.

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**Acetylcholine contractures of denervated skeletal muscle in isotonic potassium sulphate.** By D. H. JENKINSON and J. G. NICHOLLS*. *Departments of Biophysics and Physiology, University College London*

Smooth muscle can contract in response to acetylcholine even after it has been depolarized by immersion in isotonic potassium sulphate (Evans, Schild & Thesleff, 1958). Mammalian skeletal muscle acquires this property after denervation; to demonstrate this, strips of rat diaphragm, denervated 8–21 days previously, are mounted for isometric recording at room temperature. Immersion in potassium sulphate produces a transient contracture. Provided that the bathing fluid contains calcium, acetylcholine ($10^{-6}$–$10^{-5}$ g/ml.) will now cause tension development (up to 25% of the maximum response to acetylcholine in the polarized muscle). The amplitude of the response in the depolarized muscle is dependent on the calcium concentration and on temperature. The effect can be abolished reversibly by tubocurarine ($3 \times 10^{-6}$ g/ml.) and is potentiated by prostigmine ($2 \times 10^{-7}$ g/ml.).

This tension development does not appear to be associated with changes in membrane potential. There are, however, large increases in membrane

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permeability (Axelsson & Thesleff, 1959; Klaus, Kuschinsky, Lüllmann & Muscholl, 1959) which can be observed by measuring the fluxes of labelled sodium and potassium ions.

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**Sensory terminations in the muscle spindle of the frog.** By B. Katz. *Department of Biophysics, University College London*

According to Cajal (1888) and Dogiel (1890) the terminal branches of the sensory axon in the muscle spindle of the frog consist of very fine 'varicose filaments' which run along the encapsulated portion of the intrafusal muscle fibres. These terminal branches and their contacts with the muscle fibres have been studied further with the electron microscope.

In longitudinal section, the 'varicose nerve filaments' appear as chains of 'microspindles' whose bulbous expansions contain numerous small mitochondria and may reach up to 2 or 3 $\mu$ in diameter. They taper off longitudinally into cylindrical connecting tubes of as little as 0·15 $\mu$ diameter. The surface of the intrafusal muscle fibres has cup-like depressions in which many of the sensory nerve bulbs are seated in close contact with the muscle surface. There is a gap between the two cells, about 150 Å wide, but this is bridged here and there by very fine filaments or short processes of one cell closely approaching or touching the other. Suppose that, during stretch, the concavities of the muscle surface become shallower and thereby tend to separate from the convex surface of the nerve. The mechanical stimulus would then be applied to the axon membrane through points of adhesion or of intimate contact between the two cell surfaces.

In most intrafusal muscle fibres the region of sensory contacts extends over a length of several hundred microns. This region can be divided into two types of morphologically quite distinct zones: (*a*) at either end of the region, there is a 'compact' zone about 200–300 $\mu$ long (in 'slack' muscle), in which the muscle fibre retains much the same cross-sectional area and number of myofilaments as is found in the extracapsular region; (*b*) a 'reticular' zone in the centre, about 50–100 $\mu$ long, in which the fibre loses some 85 % of its content of myofilaments and divides into multiple fins and branches held together by slender membrane connexions. The interstices between the splayed-out parts of the muscle fibre are filled with a dense network of fine connective-tissue filaments (approx. 50 Å thick). This extracellular network is a continuation, and local replacement, of the ordinary sarcolemmal 'basement membrane'. The regions of termination

of myofilaments, and of attachment of the supporting extracellular net at the fibre surface are marked by unusually dense patches of the cell membrane. The special features of the reticular zone are of twofold interest: (i) the extracellular network presumably gives the fibre the necessary elastic support, preventing it from being damaged during intrafusal contraction; (ii) the presence of this network and the virtual absence of a system of sliding myofilaments indicate that the local mechanical impedance to stretch differs from that of the compact zones: it probably offers less viscous and more elastic resistance. Hence, the 'dynamic' or 'velocity component' of the afferent response may originate mainly from nerve contacts within the reticular zone, and the 'static' or 'displacement' response from the compact zones.

Finally, an unusual, periodic, structure is found within the sensory portion of intrafusal muscle fibres. It consists of a longitudinal 'ladder' whose rungs are made of dense disks, 0·2–0·8 $\mu$ wide and repeating at approx. 1500 Å intervals. This structure appears to be relatively inextensible, its characteristic period varying much less with muscle length than the average length of the sarcomeres.

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**Frog's spinal cord potentials generated by impulses in cutaneous afferent fibres.** By MARIA C. BRAVO and A. FERNANDEZ DE MOLINA. *Departamento de Biofisica, Centro de Investigaciones Biologicas, C.S.I.C., Madrid, Spain*

The potential changes generated in the VIII and IX segments of the cords of decerebrated and spinal frogs, resulting from stimulation of cutaneous nerves, have been recorded through an extracellular capillary electrode by the usual method (Coombs, Curtis & Landgren, 1956; Fernandez De Molina & Gray, 1957).

By graded strengthening of stimulation there appeared progressively three phases of potential changes following the initial afferent primary spike. The first phase appeared at threshold stimulation for cutaneous fibres; it reached its peak 1·8 msec after the negative peak of the incoming volley and had a total duration of 12–14 msec. Its amplitude was maximum at 1·5 threshold, being reduced to a half at frequencies of 20/sec. Its afferents are the largest from the skin, having a conduction velocity of 25–28 m/sec.

The second phase developed at 1·5–2 times threshold; it reached its

peak at 1·8 msec after the negative peak of a second component of the afferent volley. Its total duration could not be determined with accuracy because it became obscured by the third phase. At 5 times threshold its amplitude was maximum, being reduced by repetitive stimulation in the same way as the first phase. The second spike, which had a conduction velocity of 18–20 m/sec, appeared in the action potential recorded from the dorsal root as this second phase developed.

The third phase reached its peak 20–25 msec after the incoming volley and had a decaying time of 250 msec. It developed almost parallel to the second phase but reached its maximum at higher strengths of stimulation. Frequencies of 12–15/min were enough to suppress it. The second component (Dun, 1944) of the dorsal root potential appeared at the same time as the third phase and was suppressed by the same frequency of stimulation.

When recording at various depths along tracks in a dorsoventral direction the three phases of potential had their maxima at different levels in the dorsal region of the cord. More ventrally two positive waves could be seen and sometimes a complex negative wave was superimposed on the positive ones. The incoming volley could only be seen in the dorsal half of the cord.

It may be assumed that the first and second phases are due to post-synaptic activity in second order neurones; the third phase would represent activity in neurones of a higher order.

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**An inhibition of growth and testicular development by reserpine.**

By P. Hagen. *Department of Biochemistry, University of Manitoba, Winnipeg 3, Canada*

One weekly injection of reserpine 1, 2 or 4 mg/kg, begun at 1 month of age, retarded the growth and sexual development of male chickens. The weekly increases in weight of the birds receiving reserpine were only between 50 and 60 % of those of control birds. Whereas at 3 months of age both combs and wattles of the control birds were well developed, the combs and wattles of the male chickens receiving reserpine resembled those of female birds. At autopsy at 14 weeks of age, the weights of the testes of the treated animals were only about 5 % of those of the untreated chickens. Apparently reserpine either prevents the secretion of gonadotrophic hormone by the pituitary gland or inhibits the action of gonadotrophic hormone on the testis. It is also possible that it inhibits the secretion of pituitary growth hormone.

**The reduction in the distensibility of the capacity vessels in the forearm during hyperventilation.** By D. LYTTLE. *Department of Physiology, The Queen's University of Belfast*

Pressure–volume relationships of the capacity vessels of the human forearm were studied using pressures within the normal physiological range. Volume changes were recorded plethysmographically. A cuff on the upper arm was inflated to 50 mm Hg for about 4 min; the cuff pressure was then quickly reduced to 40 mm Hg for 15 sec and subsequently down to zero by 5 mm steps each lasting for 15 sec. After 15 sec the volume at any step level remained almost unchanged for the next 4 min if the cuff pressure was maintained for this period. This 'step-down' technique was found to give results similar to those obtained by the 'step-up' method of Eckstein, Hamilton & McCammond (1958), but has the advantage that the measurements occupy less time. The change in volume, in ml./100 ml. tissue, when the cuff pressure was reduced from 25 to 10 mm Hg was selected as an index of distensibility. A mean decrease of 12% in this index of distensibility was found in sixteen subjects during over-ventilation of air or 4% $CO_2$, and when breathing 9% $CO_2$; the respiratory minute volumes were similar in the three cases. In an attempt to cause pressure disturbances within the thorax of the same order as those occurring in over-ventilation, but without an increase in tidal air, the subjects were made to breathe with resistance to air flow; this failed to bring about any significant change in distensibility. It is concluded that the reduction of distensibility of the capacity vessels observed during over-ventilation is not due to changes in the tension of $CO_2$ in the blood, and probably not to cyclical changes of pressure within the thorax. The possibility remains that it is initiated by stretch receptors in the lungs.

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**Further studies of the dissociation between action potentials and the contractile mechanism in smooth muscle.** By J. AXELSSON. *Department of Pharmacology, University of Oxford*

Previously it has been shown that spontaneously occurring action potentials in the smooth muscle of the guinea-pig's taenia coli fail to produce tension when the sodium ion is substituted by lithium or by choline (Axelsson & Bülbring, 1959)

During prolonged exposure to this abnormal ionic environment the spontaneous activity becomes gradually slower and eventually stops.

Nevertheless, for a period, the cells may still be electrically excitable and capable of conducting spikes. These produce no tension. Thus the previous results have been confirmed on electrically stimulated preparations.

Recently, hydrazine has been described as a substitute for Na by Koketsu & Nishi (1959). They found that striated muscle maintained its excitability in sodium-free hydrazine solution. In smooth muscle hydrazine, like lithium and choline, produces a dissociation between action potentials and tension response, which progressively declines while spontaneous spikes are still recorded. However, spontaneous activity stops after 10–30 min. Then there is a period, not longer than 1 hr, while the preparation still responds to electrical stimulation by conducted action potentials. The subsequent state of inexcitability or block of conduction is always reversed on re-introducing Na. These experiments suggest that smooth muscle only maintains its excitability in absolutely sodium-free medium for a limited time.

Dr H. R. Ing kindly prepared for us another substitute for NaCl, lithium-ethane sulphonate. It had been shown (Axelsson & Bülbring, 1959) that substitution of the Cl by using sodium-ethane sulphonate did not affect the tension response. In the presence of lithium-ethane sulphonate (substituting both Na and Cl) the muscle was found to maintain excitability and was able to conduct spikes for about 1 hr, but the tension response was abolished.

The experimental results are in favour of the hypothesis that in smooth muscle $Na^+$ as well as $Ca^{2+}$ plays an essential part in the activation of the contractile mechanism by an action potential.

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**Vasomotor reactions in the human forearm and hand during leg exercise.** By D. A. Blair, W. E. Glover and I. C. Roddie.
*Department of Physiology, The Queen's University of Belfast*

During leg exercise on a bicycle ergometer, blood flow in the hands and forearms of recumbent subjects was measured by venous occlusion plethysmography. In three highly practised subjects, when movement artifact was largely eliminated and emotional disturbance was minimal, exercise usually resulted in a small decrease in forearm flow and a moderate rise in arterial pressure. When vasoconstrictor fibres in the forearm were blocked by bretylium tosylate (Blair, Glover, Kidd & Roddie, 1959) or when the deep nerves to the forearm were blocked, exercise caused an

increase in flow which could be explained by the associated increase in arterial pressure. It was concluded that vasoconstrictor tone in the forearm is normally increased during exercise (Harpuder, Lowenthal & Blatt, 1957; Muth, Wormald, Bishop & Donald, 1958). Forearm blood flow did not increase during exercise when only the cutaneous nerves were blocked, indicating that the increase in vasoconstrictor tone was occurring in muscle.

During fairly severe exercise the blood flow in the forearm in which the cutaneous nerves had been blocked fell to a lower level than that in the control forearm. This result showed that reflex vasodilatation normally occurs in the skin during severe exercise and is due to an active vasodilator mechanism. Reflex vasodilatation also occurred in the hand during fairly severe exercise and was preceded by a transient vasoconstriction. Both the dilatation and constriction were mainly due to alterations in vasoconstrictor tone, since they were reduced after intra-arterial administration of bretylium tosylate to the hand. It is likely that the increase in heat production during severe exercise was responsible for the reflex vasodilatation in skin.

It has been postulated that cholinergic vasodilator fibres to muscle vessels may be activated during the early stages of exercise (Uvnäs, 1954). In the present experiments on highly trained subjects the fall in forearm blood flow was not greater in an atropinized forearm than in a control forearm. However, in subjects unaccustomed to the procedure, mild exercise often caused a slight increase in forearm blood flow and this increase was sometimes reduced by atropine. These findings suggested that vasodilator fibres to muscle may be excited during exercise, perhaps as a consequence of emotional stress (Blair, Glover, Greenfield & Roddie, 1959), but that their activation is not an integral part of the general vasomotor response to exercise.

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**Cutaneous thermoreceptors with non-myelinated afferent fibres.** By H. Hensel, A. Iggo* and Ingrid Witt. *Physiological Institute, University of Marburg (Lahn), Germany*

Thermoreceptors in the skin of the cat were examined by recording electrical activity in strands dissected from the saphenous nerve. The thermal stimuli were provided by thermodes controlled to $\pm 0{\cdot}02°$ C placed on the surface of the skin, from which the hair had been removed with strontium sulphide. Some receptors were excited by heating and others by cooling the skin and all were relatively insensitive to mechanical stimulation as compared with the C mechanoreceptors and the A fibres. The conduction velocities in the afferent fibres of some of the thermoreceptors were measured, and ranged from 0·8 to 1·5 m/sec.

Some of the receptors were sensitive to very small changes in cutaneous temperature in the physiological range. The receptors excited by *warming* the skin responded with a phasic burst of impulses when the thermode temperature was raised by as little as 0·5° C in the range 32–37° C. At thermode temperatures above 37° C the discharge persisted and was maximal about 42° C. The receptors excited by *cooling* had a steady discharge of impulses up to skin temperatures of 30–32° C, with maxima at 20–25° C. Sudden cooling by as little as 0·2° C was sufficient to raise the discharge, e.g. in one fibre the rate of firing changed from 2 to 4 impulses/sec. The dynamic sensitivity of these receptors was as great as that of both the cold receptors of the cat's tongue and temperature sense in man.

Other receptors excited by heating the skin were less sensitive to thermal stimuli in the physiological range. These receptors, which correspond to the heat receptors described by Iggo (1959), required greater thermal changes before they were excited.

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**The effect of stimulating the sympathetic nerves to the heart on the relationship between ventricular end diastolic pressure and the changes in length of a segment of ventricular myocardium.** By R. J. Linden,† J. H. Mitchell and S. J. Sarnoff. *Laboratory of Cardiovascular Physiology, National Heart Institute, Bethesda, Maryland, U.S.A.*

In the anaesthetized dog with the heart rate controlled, stimulation of the left stellate ganglion caused an increased left ventricular stroke work

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(stroke volume × mean aortic pressure) from a lower ventricular end diastolic pressure. It was not known whether this increased stroke work resulted solely from an increased contractility of the ventricle or in part from an increased extensibility of the ventricular myocardium, i.e. whether there was a change in relationship between end-diastolic pressure and 'fibre' length.

To investigate this problem an instrument was designed to record the changes in distance between two points in the ventricular myocardium. It consists of two light lever arms, each of which is attached at one end, by means of a small freely moving hinge, to the rotating shaft of a microtorque potentiometer. The other end of each lever is thrust into the myocardium. The outputs of the potentiometers are connected so that the algebraic difference between the two angles of rotation is obtained. Each potentiometer shaft rotates only when the motion of the intramyocardial part of the lever has a component along a line parallel to the line joining the centres of the two hinges. Thus the record obtained gives an index of the changes in distance between the two points in the myocardium. The records were only examined quantitatively during periods of the heart cycle when there was almost an equilibrium state between pressure and segment length, such as exists at the end of the period of diastasis.

Stimulation of the left stellate ganglion, freed so that only the nerves to the heart were intact, caused a shortening of the observed segment of the myocardium at the end of diastole as well as an increase in left ventricular stroke work and a decrease in ventricular end-diastolic pressure. The relationship between ventricular pressure and myocardial segment length at the end of diastasis was investigated over a range of ventricular diastolic pressures by repeated infusion and bleeding. It was found that there was no change in this relationship when the left stellate ganglion was stimulated.

Thus it may be inferred that no change in extensibility of the ventricular muscle results from stimulating the sympathetic nerves to the heart. So that at a given ventricular end-diastolic pressure, the increased stroke work produced by stimulation occurs from the same end-diastolic fibre length and is a result solely of an increased contractility of the ventricle.

**The absence of the blood–brain barrier from special areas of the brain.** By B. B. Brodie, E. O. Titus and C. W. M. Wilson*. *Laboratory of Chemical Pharmacology, National Heart Institute, Bethesda, Maryland, U.S.A.*

The uptake by the pituitary of basic dyes, inorganic ions and thyroxine from the blood has been described (Wislocki & King, 1936; Bakay, 1952;

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Ford & Grass, 1958), and the uptake of dyes and $^{32}P$ into the area postrema and intercolumnar tubercle has also been reported (Davson, 1956). It seemed possible that the blood–brain barrier in each of these special regions might be absent, or at least less effective than elsewhere in the brain, not only for inorganic ions but also for organic compounds.

The penetration of sulphaguanidine and *N*-acetyl 4-amino-antipyrine (*N*-AAP) into these regions has been compared with their penetration into muscle, liver and other parts of the brain in the cat after intravenous injection. Measurements of the penetration of the compounds into the tissues were made at times when the differences between their concentrations in the brain, and in plasma, muscle or liver were large. These times depended upon the rates of passage of the compounds through the blood–brain barrier and are functions of their lipoid solubility (Mayer, Maickel & Brodie, 1959). Their relative concentrations in the different areas are shown in Table 1.

The absorption of the compounds from the blood into the pituitary and area postrema was as great as their absorption into liver and muscle. The intercolumnar tubercle absorbed significantly greater quantities than the cortex, but the uptake by the hypothalamus resembled that by the cortex. Both lobes of the pituitary absorbed *N*-AAP to about the same extent.

TABLE 1

| | Tissue:plasma ratio | |
|---|---|---|
| | Sulphaguanidine | *N*-AAP |
| Liver | 0·97 | 1·14 |
| Muscle | 0·81 | 0·99 |
| Pituitary | 1·04 | 0·84 |
| Area postrema | 1·20 | 0·96 |
| Intercolumnar tubercle | 0·43 | 0·71 |
| Cortex | 0·16 | 0·33 |
| Hypothalamus | — | 0·32 |
| Time after injection (min) | 255 | 25 |

These experiments confirm the earlier observations that both lobes of the pituitary, the area postrema and the intercolumnar tubercle, differ from other parts of the brain in their power of absorbing compounds from the blood. *N*-AAP is not bound to the cellular constituents but is evenly distributed throughout the body water. The rapid entry of *N*-AAP into these tissues therefore is not due to a special affinity for the tissue cells, but is due to the complete absence of the barrier which separates blood from brain. As a result these areas can take up organic compounds from the blood as readily as can liver and muscle. It is concluded that these areas lie outside the blood–brain barrier which encloses other parts of the brain.

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**The post-excitatory recovery process in frog skin mechanoreceptors.** By W. T. CATTON. *Department of Physiology, King's College, University of Durham, Newcastle upon Tyne* 1

Brief mechanical or electrical stimuli were applied to the isolated calf skin of the frog by means of a sharp pin attached to a Rochelle salt crystal. The action potentials in single sensory fibres evoked by the stimulation of mechanoreceptor endings were recorded in the skin nerve branch. Pairs of stimuli were applied with a stimulus interval ranging from 2 to 2000 msec. Recovery curves were plotted by measuring the change in threshold for a test pulse which followed a conditioning pulse at varying intervals. With subliminal mechanical (*M*) or electrical (*E*) conditioning pulses the threshold rise was slight and lasted not more than 20 msec. After a supraliminal *M* or *E* stimulus the recovery was more prolonged, extending to about 2000 msec and divisible into three phases: (*a*) an absolute refractory period of 3–5 msec; (*b*) a rapid phase of recovery (5–15 msec); and (*c*) a slow phase (extending to 2000 msec). It was difficult to distinguish the recovery curve following an *E* stimulus from that following an *M* stimulus to the same receptor. A careful study of many results suggested that recovery was slightly more rapid in the early phase after an *E* conditioning stimulus, and that the relative delay shown in recovery from an *M* stimulus might be related to the time course of mechanical recovery of the skin surface, which was short (not more than 10 msec). Those endings propagating the largest spike responses were found to show slower recovery than endings propagating small spikes, and the rise of threshold with repetitive stimulation was greater. Some functional correlations with spike amplitude have been described previously for this preparation (Catton, 1958). Recovery curves were greatly influenced however by the following factors: (i) change of temperature; (ii) previous repetitive stimulation; (iii) age of the preparation; and (iv) increase in strength and/or duration of conditioning stimulus. A rise of temperature caused more rapid recovery; all the other factors caused delay. Tentative conclusions are as follows. The brief refractory period and prolonged recovery phase following a supraliminal stimulus applied to a mechanoreceptor ending are associated with the initiation of an impulse, since a subliminal stimulus produces only a slight rise of threshold of short duration (cf. Lindblom, 1958). This prolonged

recovery is a special feature of the ending itself, since the refractory phase associated with an impulse propagated in a myelinated axon is brief. The state of mechanical deformation of the skin has only a small effect on the excitability of a mechanoreceptor, which is limited to the early phase of recovery.

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**Changes of motor reflexes produced by tenotomy.** By GERTA VRBOVÁ.
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Modification and co-ordination of motor reflexes have so far been studied mainly in acute experiments, and little is known of the effect of chronic changes of sensory input on the function of the central nervous system. Sperry (1945) concluded that a pattern of movement, once established, is never changed by alteration of the sensory input. Later (Sperry, 1947), he found that such modification of motor reflexes can occur in the monkey. Some results of previous experiments on conditioned motor reflexes (Vrbová & Gutmann, 1956; Gutmann & Vrbová, 1959) suggested that in rabbits, rats and cats the sensory input associated with the performance of a movement is important for the maintenance of motor reflexes. The results of the present investigation provide direct evidence that motor reflexes are modified in such mammals by chronic changes of sensory input.

In the present experiments, which were performed on rabbits, the sensory input from the hind limb was altered by cutting all the tendons round the ankle joint, thus preventing any active movement. As an indicator of activity action potentials were recorded before and at regular intervals after tenotomy in the tibialis anterior muscle at rest and during reflexly elicited activity. A flexor reflex was elicited by pinching the skin of the foot, and postural reflexes by lifting the animal free of the ground or by tilting it in a specially constructed box. Up to 2 weeks after operation all the reflex responses in the tenotomized muscle were normal. During the third week the reflex responses began to decrease. They progressively diminished, and by the sixth post-operative week were practically abolished in all animals. The disappearance of these reflexes did not seem to be due to changes in the peripheral nerves, neuromuscular junction or muscle fibres since there was no change in the e.m.g. on stimulation of the peroneal nerve. Moreover, when the flexor reflex was tested in the same animal

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under anaesthesia the response of the tenotomized muscles was undiminished. In some experiments there was even evidence of reflex facilitation, in that the latent period of the reflex was decreased. Such facilitation has also been reported by Beránek & Hník (1959) for monosynaptic reflexes in cats.

Thus spinal and postural reflexes are inhibited when the animal is conscious, whereas in the same animal under anaesthesia spinal reflexes seem to be, if anything, enhanced. It therefore appears that the inhibitory influence is exerted by supraspinal structures.

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**Visual acuity, threshold flux and eye movements.** By F. H. C. Marriott and M. H. Pirenne. *University Laboratory of Physiology, Oxford*

The smallest flux of white light detectable by the dark-adapted fovea is about $2{\cdot}5 \times 10^{-12}$ photopic lumen (range $10^{-12}$ to $4 \times 10^{-12}$). This value is based on the review by Stiles, Bennett & Green (1937) and on the average *effective* area of the expanded pupil (Le Grand, 1957). It may be compared with the light flux received by the eye from the illuminated gap of a black Landolt C which can just be resolved at a given field luminance by a subject maintaining central vision. Shlaer's (1937) measurements show that it is only at acuity about 2 that this flux begins markedly to exceed the foveal threshold flux. For his subject A.M.C. the fluxes for various gap sizes were: 10′ of arc, $1{\cdot}2 \times 10^{-12}$ photopic lumen; 1·1′, $0{\cdot}7 \times 10^{-12}$; 0·87′, $0{\cdot}8 \times 10^{-12}$; 0·56′, $3 \times 10^{-12}$; 0·51′, $7 \times 10^{-12}$; 0·40′, $400 \times 10^{-12}$; the final acuity plateau corresponded to 0·38′ of arc.

A similar state of affairs exists for scotopic acuity and the smallest flux detectable by the dark-adapted periphery (Pirenne, Marriott & O'Doherty, 1957). Thus over most of the acuity range, up to a value of about 1/0·56 which is reached at a retinal illumination of 115 trolands (about $10^7$ times the absolute threshold for a large field), the flux received by the eye from the gap of the just resolvable C is of the same order as the absolute threshold flux, under conditions of dark adaptation, for the retinal region being used. (Implications of this have been discussed by Pirenne, 1957, 1958.)

Within this wide range of acuities and luminances, therefore, eye movements the extent of which is small compared with the gap, or the thickness,

of a just resolvable black Landolt C must be unable to stimulate on and off retinal units lying along the edges of the C—unless these units can respond to changes in light flux smaller than the smallest flux detectable by the relevant retinal region. (Published studies on eye movements do not seem to provide evidence that eye tremors small compared with the dimensions of the smallest detail resolvable at the luminance being used can restore vision of the object once it has faded as a result of stabilization of the retinal image).

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**The effect of low temperature upon the recovery of frog sartorius muscle from potassium depolarization.** By D. W. Grieve.
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In 1956 Csapo & Wilkie reported a curious effect of temperature on the inexcitability produced in frogs' sartorius muscles by the action of 25 mM-KCl. The onset of inexcitability follows roughly the same time course at 25° C and at 2° C. On the other hand, the recovery when normal Ringer's solution is replaced is very much slower at the lower temperature. However, if the cold recovering muscle is suddenly warmed, excitability returns equally suddenly, and further cooling to 2° C has no effect other than is expected from the usual temperature coefficients.

The experiments described above leave one in doubt whether it is the membrane potential, the action potential or the contractile mechanism that has failed to recover. I have repeated the experiments, at the same time using micro-electrodes to stimulate and to record resting potentials from superficial fibres in the muscle. The results showed that when recovery was delayed, it was a consequence of delayed repolarization; as soon as membrane potentials exceeded a level of about 63 mV, the fibres twitched normally when stimulated.

The question remains, what is it that underlies the delayed repolarization? It can be shown, by considering the muscle as a system in Donnan equilibrium (Boyle & Conway, 1941) that in Csapo & Wilkie's experiments, KCl must have entered the fibres. The present work shows that if the high-K soaking solution has a composition that prevents KCl from entering the fibres, i.e. $[K] \times [Cl]$ product the same as for Ringer's solution, or chloride replaced by the impermeant methyl sulphate, then there is no delay of repolarization that cannot be accounted for by diffusion in the interspaces.

It has further been shown experimentally that the membrane potential of the recovering muscle is far more influenced by the ratio $[Cl]_{in} : [Cl]_{out}$ than by $[K]_{out} : [K]_{in}$. This suggests that the situation resembles the one described by Hodgkin & Horowicz (1959) in experiments at room temperature, in which the permeability to potassium had dropped to a very low value, following a period of soaking in a high (KCl) solution.

The mechanism of the sudden recovery on warming remains something of a mystery, but the most likely explanation is a sudden restoration of permeability to potassium. There are a number of reasons for thinking that the KCl that has been trapped inside the fibres is not suddenly released during the period of warming.

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**Acclimatization to heat and its rate of decay in man.** By J. M. Adam, R. H. Fox, G. Grimby, D. J. Kidd and H. S. Wolff. *Division of Human Physiology, National Institute for Medical Research, London, N.W. 3*

In the present study sixteen soldiers, aged 18 to 22½ years, were formed into two teams of eight. The 4 hr daily routine consisted of alternating half-hour periods of work, performing a load-carrying task, and of resting seated, with an average energy expenditure of 100 kcal/m²/hr. Standing pulse rates, sweat production, rectal temperature and energy expenditure were measured. After training in a cool climate with a dry-bulb temperature of approximately 10° C (50° F) for 7 days, the subjects were tested once in an accurately controlled hot climate with dry-bulb temperature 35·6° C (96° F), wet-bulb temperature 31·7° C (89° F) and air speed 25·4 cm/sec (50 ft./min) (1st uniformity trial). During each of the subsequent 12 days one team (team A) performed the routine in the hot

climate to develop their acclimatization status, while the control team (team B) worked in the cool climate. Both teams were then re-examined once in the hot climate (2nd uniformity trial). After 2 days working in the cool environment the subjects were all exposed once to an even hotter climate, with dry-bulb temperature 40° C (104° F), wet-bulb temperature 32·8° C (91° F) and air speed 25·4 cm/sec (50 ft./min) (high temperature test). After 6 days without exposure to heat the subjects were again tested in the original hot-wet climate (3rd uniformity trial) and received their final exposure to this climate after a further 28 days (4th uniformity trial).

Between the 1st and 2nd uniformity trials there was an increase in sweat production ($P < 0{\cdot}001$) and a decrease in the rise in rectal temperature ($P < 0{\cdot}01$) in team A subjects during the 4 hr exposure, whereas team B showed no change. There was no evidence of any change in energy expenditure by either team. The difference between the two teams was also clearly shown in the high-temperature test, team A subjects having not only higher sweat rates ($P < 0{\cdot}01$) and smaller increases in rectal temperature ($P < 0{\cdot}02$) than team B but also smaller increases in pulse rate ($P < 0{\cdot}05$). The re-test of team A subjects after 6 days without exposure to heat revealed that they had lost a substantial part of the improved tolerance to heat, and 28 days later the decay in acclimatization was virtually complete. The results show that the acclimatization to heat induced as described was relatively rapidly lost when exposure to heat ceased.

## Further observations on the location of renin in the rabbit kidney.

By W. F. Cook. *Department of the Regius Professor of Medicine, Radcliffe Infirmary, Oxford*

Cook & Pickering (1959) have shown that glomeruli separated from the kidney cortex contain more renin per mg of nitrogen than the remaining non-glomerular tissue. Also, glomeruli which retain a capsule and fragments of proximal tubule and arterioles contain more renin per mg of nitrogen than glomeruli without this additional tissue. These results are consistent with the hypothesis that renin is located in the cells at the vascular pole of the glomerulus (Goormaghtigh, 1944). They are also, however, consistent with the view that the glomerular capillary and capsular epithelium contain renin (Nairn, Fraser & Chadwick, 1959). It was thought that if a sufficient number of glomeruli could be bisected into a vascular-pole half and a distal half, the distribution of renin between the halves could be determined. An even distribution would support Nairn and colleagues, whereas if renin were located at the vascular pole, only the half including it would contain renin. A suspension of magnetic

iron oxide was infused into the renal arteries of freshly killed healthy young rabbits. The glomeruli from the peripheral region of the cortex were then separated magnetically (Cook & Pickering, 1958). Glomeruli, which had the afferent arteriole filled with oxide and possessed a capsule and sometimes a short length of proximal tubule, were picked up with a micropipette. Several hundred such glomeruli were transferred to a miniature guillotine where each was bisected and the halves collected into two lots, one of which included all the vascular-pole halves and the other all the distal halves. Throughout this procedure, which took about 10 hr, the glomeruli before and after bisection were suspended in Krebs's solution at approximately 5° C. Saline extracts of each collection of half glomeruli were assayed on the rat's blood pressure as previously described. Six experiments have been done and in every case renin was readily demonstrated in extracts of the vascular-pole halves but could not be detected with certainty in extracts of the distal halves. It is concluded that renin is probably located in a structure at the vascular pole of the glomerulus but precisely which cells are involved remains to be determined.

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## Aerobic energy production and the stimulation of active sodium transfer across isolated frog skin by neurohypophysial extract.

By G. H. Wright. *Department of Physiology, St Mary's Hospital Medical School, London, W.2*

The increase in active sodium transport by isolated frog skin following administration of a maximal dose of neurohypophysial extract varies considerably among different skins of the same variety.

Leaf & Renshaw (1957) showed that neurohypophysial extract failed to stimulate active sodium transport in anoxic skins. They concluded the action of neurohypophysial hormones on ion transport is 'somehow associated with their ability to increase the availability of aerobic energy sources for operation of the ion transport mechanism'.

It was decided in view of the above finding to investigate the relation between the magnitude of the response to a maximal dose of neurohypophysial extract (Pituitrin; Parke, Davis and Co. Ltd, Batch no. LY 616A) and the aerobiosis of the skin.

The experiments were carried out during the months of May, June and July, using frogs kept in captivity for 3 or 4 months. Active $Na^+$ transport was measured on the short-circuit current principle (Ussing & Zerahn, 1951) by means of a continuous recording technique (Wright, 1959). The Ringer's solution used had the following composition (mM): NaCl 115, $CaCl_2$ 1·4, $KHCO_3$ 2·5. The pH of this solution was adjusted to 7·8.

After a 2 hr period of equilibration the skins were subjected to total anoxia for about 30 min, then quickly restored to aerobic conditions. About 30 min later the Pituitrin was administered.

Measurement showed a highly significant positive correlation ($r = 0{\cdot}763$, $P < 0{\cdot}001$) between the magnitude of the response to Pituitrin and the magnitude of the aerobic component of the short-circuit current (defined here as the difference in values of the steady short-circuit current under aerobic and anaerobic conditions). The equation of the regression line is $y = 0{\cdot}787x + 4{\cdot}4$.

From these results it is concluded that the degree of response of the ion-transport mechanism to a maximal dose of neurohypophysial extract is dependent upon the functional state of the aerobic energy source at the time of administration of the extract.

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**Development of monosynaptic paths following changed motoneurone function.** By J. C. Eccles, Rosamond M. Eccles and F. Magni. *Department of Physiology, Australian National University, Canberra, Australia*

After reviewing an extensive literature Sperry (1945) concluded that in mammals nerve cross-union did not change the simplest reflex pathways. In a re-investigation, monosynaptic activation of motoneurones by Ia afferent fibres from muscle was used as a test for plastic change. In eleven kittens (1–25 days old) the nerves to the peroneal (Per.) muscles (peroneus longus, brevis and tertius) and to the medial gastrocnemius (MG) were cross-united in aseptic operation. Twenty-one to thirty-six weeks later the motoneurones supplying these and related muscles were identified and their monosynaptic connexions investigated by intracellular recording (cf. Eccles, Eccles & Lundberg, 1957). Physiological test established that motor axons originally supplying Per. muscles regenerated only to MG

muscle, and conversely with the original MG motor axons and Per. muscles. As expected for young kittens, chromatolysis destroyed more than half of the neurones with severed axons.

Monosynaptic connexion to the original Per. motoneurones was assumed only when the central latency of the excitatory post-synaptic potential (EPSP) was less than 1·1 msec. Such EPSP's were produced by the lowest threshold (Ia) afferent fibres. Of the 125 Per. motoneurones examined intracellularly, 42 had monosynaptic connexions from the physiological extensor muscles of ankle and digits (lateral gastrocnemius, LG, 37; MG, 1; plantaris, Pl. 8; and flexor digitorum longus, FDL, 14). By contrast only 6 of 51 normal Per. motoneurones had such aberrant connexions (LG, 1; MG, 4; Pl, 1; and FDL, 2). Thus with the exception of MG there was a large increase in such monosynaptic paths, particularly from LG, where the incidence of aberrancy rose from 2% to 30% of Per. motoneurones. The largest EPSP was 1·4 mV, but most were small, the mean size for LG being 0·4 mV. Post-tetanic potentiation was abnormally large, the mean EPSP from LG being then 0·9 mV. Changed function of other motoneurones in six operations cross-uniting different nerves revealed no increase in aberrancy of monosynaptic connexions, but there was much less proximity than with Per. and LG nuclei, which are in close apposition throughout the L7 segment.

Possibly the new monosynaptic paths are due to the sprouting of fibres that is effected by the degenerated central terminals of the chromatolysed ganglion cells (cf. McCouch, Austin, Liu & Liu, 1958). But the preponderance of LG connexion suggests the operation of a more specific influence; on account of their innervation of MG muscle the Per. motoneurones specially attract Ia synapses from the most closely related synergist, LG, and to a less extent from Pl and FDL. Both the chromatolytic loss of many Ia fibres from MG, and its changed function, would account for the decline in its aberrancy. The operation of a specific influence is further suggested by the absence of aberrant connexions from the functionally unrelated hamstring muscles, despite the proximity of many hamstring motoneurones to Per. motoneurones in the $L_7$ segment. If the postulated specific influence can be substantiated by further experiment, it would provide the basis for explanations of the development of specific pathways in the central nervous system, but the manner of its operation would raise fundamental problems.

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**2, 4–Dinitrophenol and the chloride content of rat auricle.** By J. F. LAMB. *Department of Veterinary Physiology, University of Edinburgh*

The older analytical literature (e.g. Lohmann & Weicher, 1934) showed that the chloride content of heart muscle was greater than that of skeletal muscle, and recent electrical experiments (Hutter & Noble, 1959) suggested the hypothesis that the chloride equilibrium potential ($E_{Cl}$) was less than the resting potential. The following additional evidence is advanced in support of this hypothesis:

(1) The chloride content of normal left auricles after 15 min perfusion with Tyrode solution (Cl 142 m-equiv/l.) was $61{\cdot}5 \pm 1{\cdot}6$ (S.E.; $n = 9$) m-equiv/kg wet tissue (Na $59{\cdot}8 \pm 2{\cdot}1$, K $74{\cdot}1 \pm 1{\cdot}2$ m-equiv/kg wet tissue). With a sucrose space of $25{\cdot}1 \pm 0{\cdot}7$ (S.E.; $n = 10$, water 78%) the calculated intracellular chloride $[Cl]_i$ content was 43 m-equiv/kg fibre water. For passive distribution at a resting potential of 80 mV the expected $Cl_i$ is 7 m-equiv/kg fibre water. As these auricles were active the membrane potential was only at 80 mV for 95% of the total time. The membrane potential during the rest of the time would favour passive accumulation of chloride by the cells and this might partially or wholly explain the results.

(2) Perfusion of another series of auricles with Tyrode solution containing 13 $\mu$M of 2, 4–dinitrophenol (DNP) for 15 min produced a loss of chloride of 9·0 m-equiv/kg wet weight ($P < 0{\cdot}001$). This is consistent with the hypothesis that chloride is actively maintained within heart cells. A comparison between auricles perfused with normal Tyrode solution or Tyrode containing DNP (Lamb, 1959) for 15 min shows that DNP causes a loss of 12 m-equiv of K and 9·0 m-equiv of Cl, and a gain of 3·4 m-equiv of Na per kg wet tissue.

From these results the calculated $E_{Cl}$ is 32 mV and this means that Cl ions are only available to repolarize the membrane during the initial part of repolarization. This was confirmed experimentally by replacing the Cl of Tyrode solution with benzene sulphonate, when a marked prolongation of the initial part of repolarization of the action potential occurred. It was found that iodide was taken up to the same extent ($58{\cdot}8 \pm 0{\cdot}5$ m-equiv/kg wet tissue, after 30–60 min perfusion) as chloride, and under these conditions there was an acceleration of the initial part of repolarization with the appearance of a marked inflexion at about 30 mV.

After 15 min of treatment with DNP the action potentials of rat auricle (Lamb, 1959) only last some 3 msec and the resting potential has dropped 10 mV. In similar experiments in which the chloride in the Tyrode solution was replaced by benzene sulphonate the action potential only shortened to 20 msec with no drop in resting potential, or decrease in excitability.

A suggested hypothesis for the effects of DNP on heart tissue is that there is a large increase in the chloride permeability, this tending to lower the resting potential, and hasten repolarization of the action potential, towards the chloride equilibrium potential.

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**A new method for determining the half-saturation of the facilitated transfer of glucose across the human erythrocyte membrane and for studying the effect of inhibitors.** By A. K. SEN and W. F. WIDDAS. *Department of Physiology, King's College, London, W.C. 2*

When human red cells previously incubated in saline containing 76 mM glucose are resuspended in a similar medium containing less than 16 mM glucose they first swell osmotically and then shrink as glucose is lost from the cell interior. Because of saturation on the inside of the membrane about two-thirds of the exit is at a steady rate and a linear record is obtained with the photoelectric apparatus (Widdas, 1953*a*) used to follow the volume change.

By observing the 'exit' times into separate media containing glucose in the range 0·8–12·2 mM it is possible (i) to find, by extrapolation, the time for exit into a glucose-free medium ($t_0$), and (ii) to deduce the concentration of glucose which would cause the exit time to be twice $t_0$. From kinetics previously described (Widdas, 1954; Bowyer & Widdas, 1958) this last concentration is equal to the half-saturation concentration ($\phi_g$).

The advantages of this technique over that using competition, e.g. with sorbose (Widdas, 1954) are due to the faster rates of glucose exit compared with sorbose entry or exit. Slow cell-volume changes due to other causes are thus less important and the results are more reproducible. The value of $\phi_g$ obtained by this technique at pH 7·4 and 37° C is $4{\cdot}0 \pm 0{\cdot}24$ mM (or 72 mg glucose/100 ml.).

Le Fevre (1954), using glucose exit measurements in the presence of a competitive inhibitor (Phloretin), deduced the half-saturation constant to be about 7 mM. Competition with sorbose suggested a value of 10 mM (Widdas, 1953*b*).

When glucose 'exits' occur into media containing different glucose concentrations (but always containing a constant amount of inhibitor) it is also possible to obtain (i), by extrapolation, a time for exit from the partially

inhibited cells into a glucose-free medium ($t_0'$), and (ii) a concentration of glucose which doubles this exit time. If the inhibitor is a competitive one, the concentration of glucose which gives an exit time equal to twice $t_0'$ should be greater than the half-saturation constant ($\phi_g$) and this has been confirmed for the competitive inhibitors phloretin and polyphloretin phosphate.

In cells treated with 2,4-dinitrofluorobenzene (DNFB) the concentration of glucose which doubled $t_0'$ was not greater than $\phi_g$. It was known that inhibition by DNFB was irreversible (Bowyer & Widdas, 1958) but this additional evidence rules out the possibility of there being a competitive element in the action of this inhibitor.

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**The response of pulmonary arterial baroreceptors to alterations in pulmonary arterial pressure.** By J. C. G. Coleridge and C. Kidd. *Department of Physiology, School of Medicine, University of Leeds*

Recently impulses have been recorded from baroreceptors in the pulmonary artery of the cat (Swan & Whitteridge, 1956; Bianconi & Green, 1959) and the dog (Coleridge & Kidd, 1959). There is some evidence that reflex cardiovascular changes may be initiated by alterations in pulmonary arterial pressure (reviewed by Heymans & Neil, 1958). But evaluation of the normal role of the pulmonary arterial baroreceptors is hindered by an absence of information about the relationship between their impulse activity and the blood pressure in the pulmonary artery.

Afferent impulses were recorded from pulmonary arterial baroreceptor fibres dissected from slips of the cervical vagus in anaesthetized dogs with the chest open. In some experiments mean pulmonary arterial pressure was measured with a saline manometer; in other experiments pulsatile pressures were recorded with an optical manometer. Pulmonary arterial pressure was altered by means of infusions or bleeding, or by occlusion of the inferior vena cava.

When pulmonary arterial pressure was lowered, the pressures at which impulse activity disappeared in different fibres were within a narrow range (mean pressure 10–15 mm Hg; pulsatile pressure, 16/7–23/11 mm Hg). These values lie towards the lower end of the range of pressures variously quoted for the normal pulmonary arterial pressure in the dog.

When pulmonary arterial pressure was raised in steps from the threshold pressure up to a mean pressure of about 35 mm Hg, the pulmonary baroreceptors signalled each rise in pressure with an increased frequency of discharge; within this range of pressures there was an approximately linear relationship between peak frequency of discharge and the number of impulses per heart beat. Peak frequencies of 200–300 impulses/sec occurred with pulsatile pressures of about 50/25 mm Hg.

Further experiments were carried out immediately after the animal had been killed. The main pulmonary trunk was cannulated and the lobar arteries ligated. Baroreceptor activity was then examined while pressure in the isolated artery was varied by means of a reservoir connected to the cannula. Pulsatile pressure was a more effective stimulus to pulmonary arterial baroreceptor discharge than was a steady pressure. This is similar to results obtained on carotid sinus baroreceptors (Ead, Green & Neil, 1952).

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## A study of the calcium exchange in the isolated turtle ventricle.

By Ethyl Cosmos and J. G. Henrotte. *Department of Physiology, School of Medicine and Dentistry, Rochester (N.Y.), U.S.A.*

Studies done *in vitro* with $^{45}Ca$ show the occurrence of a small exchangeable portion of the total cellular calcium in resting frog's skeletal muscle (Harris, 1957; Cosmos, 1958).

We have performed similar experiments with isolated turtle ventricles (*Pseudemys elegans*) and frog's skeletal muscles of hind legs. The *in vitro* preparations of ventricles have been done according to a technique similar to Hajdu's (1958) and bathed for several periods of time in a Boyle–Conway solution containing $^{45}Ca$. The total Ca content expressed as m-equiv/kg of fresh tissue and the radioactivity expressed as the ratio of the specific activity of the tissue (in counts/min/m-moles of Ca) to the specific activity of the bathing solution (i.e. the percentage of tissue Ca exchanged with the $^{45}Ca$ of the medium) have been determined according to the methods of both Gilbert & Fenn (1957) and Cosmos (1958).

The total calcium content of turtle ventricles and frog's skeletal muscles, fresh or perfused, is equal respectively to an average of $2{\cdot}22 \pm 0{\cdot}66$ and $3{\cdot}46 \pm 0{\cdot}80$ m-equiv/kg. After 1 hr of perfusion, the ventricles exchange

65·5 ± 5·4 % of their calcium (7 experiments). When stretched by filling of liquid under a pressure of 15 cm of water, they exchange 81·2 ± 3·1 % of the calcium (9 experiments) and their contractile strength is also very much increased. Stimulation at the rate of 24/min, and in isometric conditions, does not modify those results (Fig. 1). On the contrary, frog's skeletal muscles perfused for several lengths of time exchange steadily only 21·8 ± 1·45 % of their calcium. As previously described (Cosmos, 1958) stimulation increases slightly their calcium exchangeability (28·3 ± 0·96 %). The time course of the Ca exchange (Fig. 1) shows that maximal values are almost reached already after 15 min of experiment.

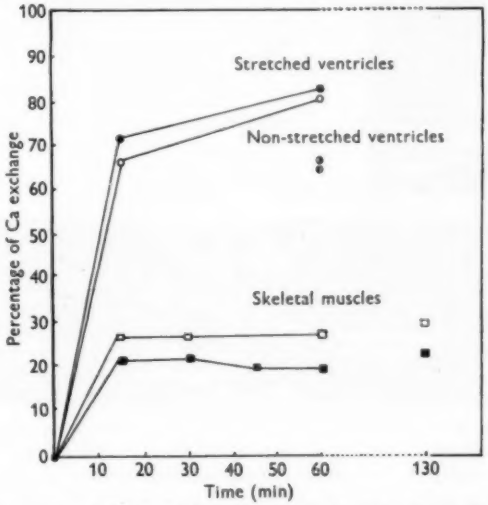


Fig. 1. Time course of $^{45}Ca$ uptake. Abscissa: time in the radioactive fluid. Ordinate: percentage of muscular calcium exchanged with the $^{45}Ca$ of the fluid. ○, Stimulated; ●, resting stretched turtle ventricles; ◑, stimulated; ◐, resting non-stretched ventricles; □, stimulated; ■, resting frog skeletal muscles.

The linkage of the largest portion of the cellular Ca is, thus, much looser in the ventricle than in the skeletal muscle. This phenomenon could explain the well-known dependence of the cardiac contractile strength upon the presence of calcium in the extracellular fluid (Salter & Russel, 1951; Niedegerke, 1957).

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**The site of origin of the efferent action potentials in the giant nerve cell of *Aplysia*.** By L. Tauc. *Centre d'Études det Physiologie nerveuse, C.N.R.S., Paris, France*

It was shown (Tauc, 1960) that in the giant nerve cell of *Aplysia*, inactivation or removal of the cell body does not affect the synaptic transmission. Then we tried to get direct evidence that, even in normal conditions, the efferent impulse takes its origin from an axonal region near the soma, this latter being excited only by way of antidromic propagation.

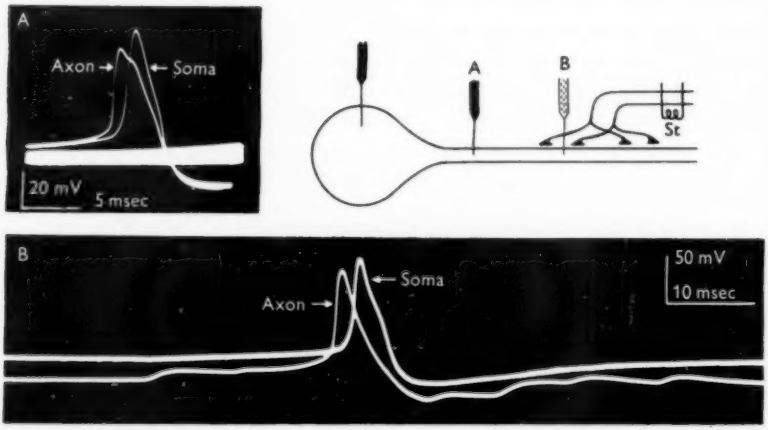


Fig. 1. Simultaneous intracellular recordings from the soma and axon of the giant cell of *Aplysia*, stimulated orthodromically (the stimulus artifact in B is outside the record). The distance of the axonal micro-electrode is 350 $\mu$ in A, 2 mm in B; A and B being taken from different neurones. Excitatory post-synaptic potentials can be seen on the record taken from the axonal region in B.

Our experimental procedure was to introduce a micro-electrode into the axon near the soma and another into the cell body so as to be able to observe in both structures simultaneously the time sequence of action potentials induced by different modes of excitation and also during spontaneous activity. It thus appeared that the axonal spike always precedes the somatic one, even when a direct stimulus is applied to the soma by means of a third intracellular micro-electrode. Fig. 1 A and B shows two cases of synaptically induced spikes in which the sequence is obvious.

When the record is taken at some distance from the soma (about 2 mm in B) post-synaptic potentials are visible, whereas no sign of these can be seen on the somatic record. This observation shows that synaptic contacts are distributed along the axon in the neuropile and relatively far from the cell body.

A double response is often recorded in the proximal part of the axon following antidromic stimulation, when the delay between the axonic and somatic spikes reaches several msec. In this case, the proximal part having recovered from its refractory period, a recurrent response can be initiated and two successive spikes are recorded.

These findings, together with extracellular observations, allow us to conclude that in the giant cell of *Aplysia* a functional discontinuity is present somewhere between the soma and the axon; this part of the axon appears to have a threshold lower than that of the cell body so that the efferent action potential originates in this region. Finally, it is this latter part of the axon which acts as a pace-maker when spontaneous activity is present.

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PROCEEDINGS OF
THE PHYSIOLOGICAL SOCIETY

BIRMINGHAM UNIVERSITY MEETING

22–23 *April* 1960

**A method for studying the rate of blood flow through the bone marrow of a rabbit's femur.** By J. D. CUMMING. *Department of Physiology, University of Birmingham*

Drinker & Drinker (1916) perfused the tibial marrow of dogs to demonstrate responses to adrenaline and to electrical stimulation of nerves. Since that time no direct methods for the study of the blood flow through bone marrow appear to have been described.

The femoral shaft of a rabbit contains red marrow even in the adult animal, and has no cancellous bone running across the myeloid canal. A single distinct vascular bundle can be seen running from the femoral vessels to the bone. Injections of dye show that the nutrient vessel entering the shaft supplies all the marrow except for a very small amount in the epiphyses at either end of the bone. These two sites receive blood from the articular branches of the hip and knee joints.

Anaesthesia is maintained with an ether–oxygen mixture through a tracheotomy tube after induction with intravenous pentothal. One carotid artery is cannulated to obtain a record of the blood pressure. A skin incision is then made on the inner aspect of one thigh parallel and a little lateral to the femoral vessels. The skin is retracted widely, and a pad of fat and mammary tissue lying superficial to and concealing the inguinal ligament is divided between ligatures. The femoral canal is opened, the vessels and nerve exposed, and cleaned of their investing connective tissue. It is necessary at this stage to divide the superficial inferior epigastric vessels. To obtain adequate exposure of the proximal end of the femoral vessels the inguinal ligament is divided laterally and stripped off the femoral canal. The profunda and circumflex branches are next ligated. Other small inconstant muscular branches are then sought and tied. The only small vessels left intact are the nutrient artery and vein. These leave the under side of the femorals and run obliquely to the femur ensheathed in fat and areolar tissue. The point of junction of the nutrient vessels with the femoral ones is variable but is always in the upper one third of the latter.

The next stage of the operation is the most difficult and consists of separating the femoral artery and vein high in the groin, and passing a ligature around the latter. Division of the femoral nerve in its lower one third and the passing of ligatures distally around the femoral artery and vein complete the preparation.

After heparinization of the rabbit the femoral vein is tied above and below the entrance of the nutrient vein. The femoral artery is ligated in its lower third to prevent infarction of the limb. The femoral vein is then cannulated and the volume of effluent from the nutrient vein is measured with a drop recorder. Constant basal rates of flow are then recorded by using a Thorp impulse counter.

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**Frequency modulation of tonal stimuli by means of voltage-sensitive capacitors.** By I. C. WHITFIELD. *Department of Physiology, University of Birmingham*

In the study of the dynamic properties of the auditory system it is useful to employ sound stimuli, other than clicks, which are nevertheless of well defined composition. Frequency modulated tonal stimuli of constant intensity fulfil this role well (Whitfield, 1953, 1957). In previous work (Whitfield, 1957) mechanical methods of modulation were employed, with consequent limitations on the flexibility with which the modulation envelope could be changed. It is more convenient to use a voltage of appropriate wave form to determine this envelope. The standard methods used in audio modulation of radio frequencies are, however, not very suitable for use at the rather low frequencies required here.

The present equipment employs a conventional phase-shift audio oscillator in which the resistance–capacitance networks incorporate semi-conductor capacitors. These devices change their capacitance with applied voltage in a sufficiently linear manner over a small range, and hence by applying a suitable low-frequency wave form the appropriate modulation is obtained. The equipment is being used to obtain modulation depths of $\pm 5\%$ with sinusoidal, and with various other simple geometric envelope shapes.

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**The rapid quantitation of silver grains in autoradiographs of tissue sections.** By A. W. ROGERS. *Department of Anatomy, University of Birmingham*

Attempts to estimate the relative concentrations of a radioactive isotope in different regions of a tissue section from its autoradiograph depend on counting the grains of developed silver in the photographic emulsion overlying those regions. Counting silver grains visually is very time-consuming, and several types of automatic grain counter have been designed. Most of these require separation of the emulsion from the tissue section, with consequent loss of accurate correlation between the histological structures and their autoradiographic image.

Gullberg (1957) has described a grain counter based on the use of indirect illumination of the slide. In this system, the silver grains, which have a high reflectivity, appear as bright specks of light on a black background, while the underlying tissue section does not reflect the light.

The apparatus to be shown makes use of indirect illumination, but is very much cheaper and simpler than that described by Gullberg. A photomultiplier tube (E.M.I. type 9524 B.), mounted in a modified camera attachment, views the autoradiograph through a microscope fitted with a standard incident illuminator. The current flowing across the photomultiplier is proportional to the light output of the microscope field under observation, which is determined by the number and size of silver grains there. Since it may be taken that the grain size is relatively constant on any one slide, the apparatus gives a rapid and accurate measure of the relative grain count over different regions of the section.

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**Left side perfusion of isolated rabbit heart.** By L. D. ABRAMS. *Department of Surgery, Queen Elizabeth Hospital, University of Birmingham*

This preparation gives a good visual demonstration of the action of the left ventricle; the relationship between the force of the left ventricle and the perfusion pressure is easily seen. The efficiency of cardiac massage in restoring coronary perfusion can be demonstrated as well as the effects of temperature change and drugs.

Satisfactory e.c.g.'s can be taken from any part of the heart.

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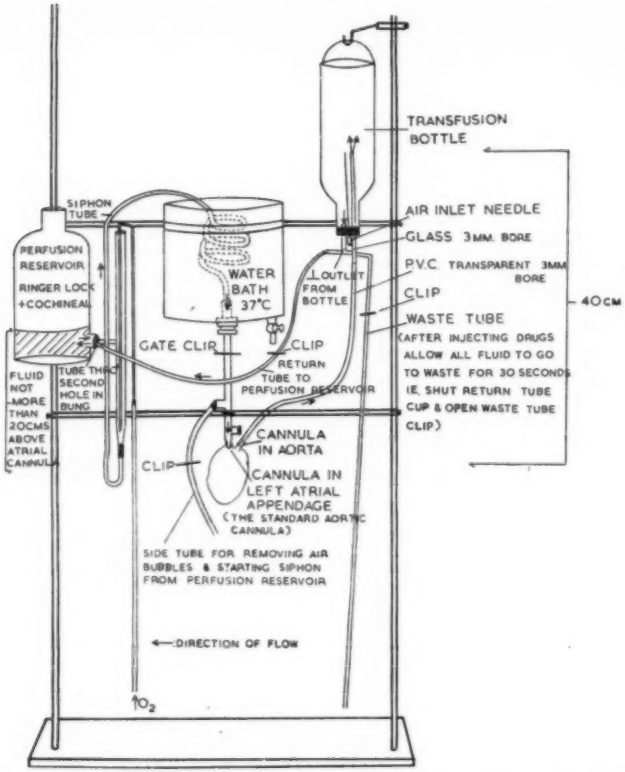
TRANSFUSION BOTTLE
SIPHON TUBE
AIR INLET NEEDLE
GLASS 3MM. BORE
PERFUSION RESERVOIR
RINGER LOCK +COCHINEAL
WATER BATH 37°C
OUTLET FROM BOTTLE
P.V.C. TRANSPARENT 3MM BORE
CLIP
40CM
WASTE TUBE
(AFTER INJECTING DRUGS ALLOW ALL FLUID TO GO TO WASTE FOR 30 SECONDS IE. SHUT RETURN TUBE CUP & OPEN WASTE TUBE CLIP)
GATE CLIP
CLIP
RETURN TUBE TO PERFUSION RESERVOIR
TUBE THRO' SECOND HOLE IN BUNG
FLUID NOT MORE THAN 20CMS ABOVE ATRIAL CANNULA
CANNULA IN AORTA
CLIP
CANNULA IN LEFT ATRIAL APPENDAGE (THE STANDARD AORTIC CANNULA)
SIDE TUBE FOR REMOVING AIR BUBBLES & STARTING SIPHON FROM PERFUSION RESERVOIR
←DIRECTION OF FLOW
$^{1}O_2$

Fig. 1. Standard Palmer isolated rabbit heart perfusion apparatus. Left side perfusion of isolated rabbit heart: 1. Set up apparatus completely and make sure siphon to atrial cannula is bubble free. Close gate clip. 2. Anaesthetize rabbit. 3. Heparinize rabbit (1000 units). 4. Open chest. 5. Remove heart, great vessels and lungs *en bloc*. 6. Ligate each lung hilum with strong thread. Leave ends long. 7. 'Nick' main pulmonary artery. 8. Open aorta 1 cm from origin after passing strong ligature round it. Insert aortic cannula and tie ligature. 9. Tie one lung hilum ligature round crossbar to support heart. 10. Make tiny incision in left atrial appendage and tie in atrial cannula. 11. Tie second hilum ligature round crossbar and adjust first. Cut off lungs. 12. Open gate clip. Start perfusion with cardiac massage.

**A microscopical study of the effect of relaxin in modifying the action of oestradiol and progesterone on the uterine tract of mice.** By KATHLEEN HALL. *Department of Physiology, University of Birmingham*

Hall (1957), and Steinetz, Beach & Kroc (1959) have found that in rats and mice ovariectomized during pregnancy administration of relaxin reduced the daily requirements for progesterone and had a beneficial effect

on parturition which was apparently not confined to its action on the symphysis pubis. These observations, and the reports by Sawyer, Frieden & Martin (1953) and Krantz, Bryant & Carr (1950) that relaxin has a quieting effect on myometrial contractions, have led to clinical trials, of relaxin in cases of threatened abortion and control of labour patterns, the results of which are conflicting.

As part of an investigation to determine whether any action of relaxin could be detected on the female reproductive tract at the microscopic level ovariectomized and young intact mice were injected for 8–9 days with combinations of 1·5 $\mu$g/day oestradiol, 0·5 or 1·0 mg/day progesterone and varying doses of relaxin, and the different parts of the uterine tract studied by means of histological and histochemical techniques. Special attention was paid to cytoplasmic nucleic acids in relation to growth changes; alterations in glycogen and other polysaccharides; and changes in alkaline phosphatase activity.

In the uterine horn relaxin produced augmentation of the action of oestradiol on myometrial glycogen and alkaline phosphatase activity and on oedematous transformation of the endometrial stroma, with deposition of quantities of acid mucopolysaccharides. Progesterone partly or completely prevented the synergistic action of the other two hormones on myometrial glycogenesis and on oedematous changes in the endometrium, but not on alkaline phosphatase activity in the myometrium and, probably, epithelium.

In the vagina relaxin and oestradiol had a synergistic effect on cellular hypertrophy and keratinization of the epithelium, and relaxin partly overcame the mucifying action of progesterone. Relaxin seemed to produce increased alkaline phosphatase activity in the lamina propria. No hormonally controlled acid mucopolysaccharides were detected in the cervix, and the techniques used did not provide a complete explanation of relaxin-induced softening of the cervix.

When injected alone, relaxin was without effect.

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**Autoradiography of tissues containing water-soluble radioactive isotopes.** By K. BROWN-GRANT, G. PETHES and A. W. ROGERS.
*Departments of Physiology and Anatomy, University of Birmingham*

Location of water-soluble isotopes in tissue sections by autoradiographic stripping film technique is technically difficult and the failure rate is high (Doniach & Logothetopoulos, 1955). Radio-iodide ($^{131}I$) has recently been shown to be concentrated in the skin of the rat *in vivo* (Brown-Grant & Pethes, 1959) and the study of its distribution in this organ has been greatly facilitated by an alternative method using the newly introduced Ilford K5 nuclear emulsion.

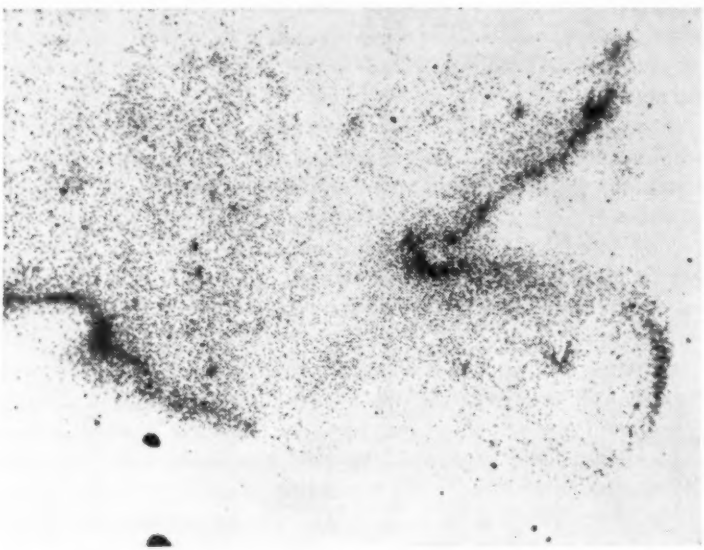

Fig. 1. Autoradiograph of a section of skin from a 2-day-old rat 2 hr after the injection of $^{131}I$. This section was mounted directly on the photographic emulsion. The line of more intense blackening corresponds to the epidermis. Magnification, × 70.

Gelatin-subbed slides were treated in the dark (Wratten No. 2 Filter at 1 m) with emulsion melted at 45–50° C, either by applying it to them with a fine artist's paint brush or by dipping them. The slides were allowed to dry at room temperature. Ribbons of sections of freeze-dried skin containing radio-iodide were mounted directly on the emulsion and gently flattened. Alternatively, the sections were mounted without dewaxing on a separate slide and held in close contact with an emulsion-covered slide by

rubber bands during the exposure period. Processing was carried out in Ilford ID 19 developer and 30 % (w/v) aqueous thiosulphate solution.

The resolution obtained by the first method was good (Fig. 1) but the sections were lost during development, and comparison could only be made

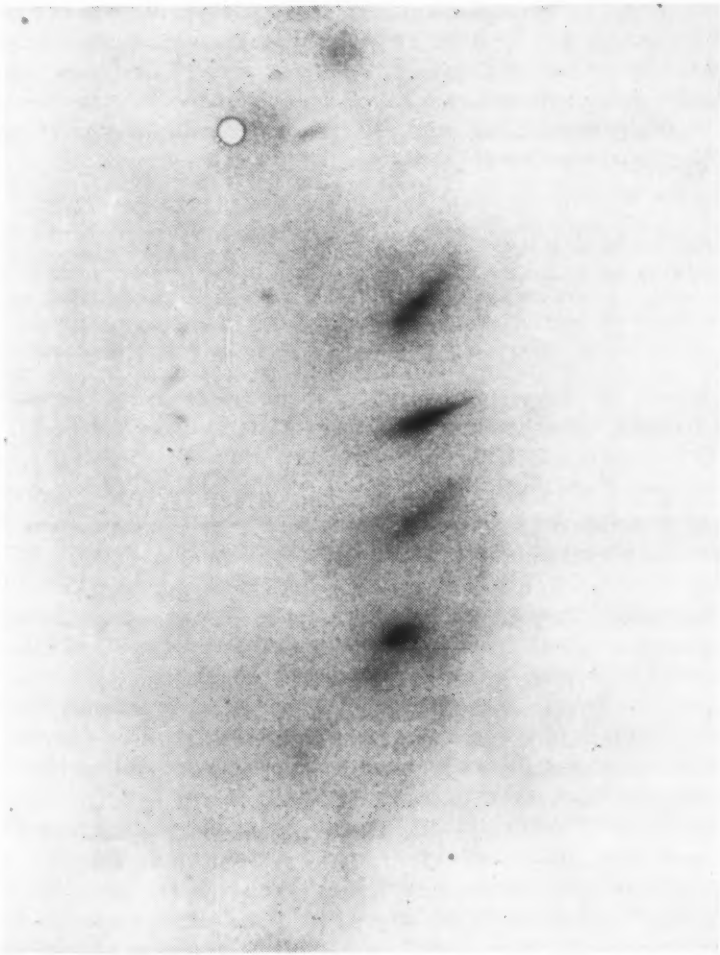

Fig. 2. Autoradiograph of a section of skin from a 10-day-old rat 2 hr after the injection of $^{131}I$. This section was mounted on a separate slide, and held in contact with the emulsion-coated slide during exposure. As well as a line of blackening which corresponds to the epidermis, some hair follicles also give an intense autoradiograph. Magnification, × 25.

with adjacent sections from the same block of tissue. In the second method resolution was not so satisfactory (Fig. 2), but the corresponding tissue sections were available for staining and examination. The results obtained by the two methods were complementary. In the skin of the rat radio-iodide was found to be concentrated in the epidermis and in the hair follicles (Brown-Grant, Pethes & Rogers, 1960). The use of emulsion was found to be much simpler than the use of stripping film and far less time-consuming, while the failure rate was low.

The necessary apparatus and the technique for handling the emulsion will be demonstrated and other examples of the application of liquid emulsions in autoradiography (Rogers, 1959) will be shown.

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**A method of investigating the effects of induced emotional changes upon gastric secretion of hypnotized subjects.** By C. Entwistle, E. H. L. Harries and M. Sim. *Departments of Physiology and Psychological Medicine, The Medical School, Birmingham*

Various studies of the effects of emotions on gastric secretion have been done in the past. The best known are those of Beaumont (1834) and Wolf & Wolff (1943), who had the opportunity of studying human beings with gastric fistulae. The classical work of Cannon (1934), demonstrating his emergency theory, is in conflict with the general belief that chronic anxiety is responsible for over-secretion of acid by the stomach.

It was found that a greater degree of control could be exercised in these studies by means of artificially induced mood changes. This was done by Wittkower (1935), in hypnotically induced fear states, and by Gordon & Chernya (1940), who used four gastric fistula cases.

Eichhorn & Tracktir (1955) produced emotional changes in subjects who had been hypnotized by eye-fixation methods, using the 'word stimulus' method as employed by Gidro-Frank & Bull (1950).

In the present series the technique used was that of suggesting to the subject that a traumatic situation was actually occurring. This traumatic situation produced the required emotion such as anger, resentment, or fear. For example, the suggestion that the subject was unjustifiably imprisoned in a small cell would cause intense resentment.

The subject, usually a medical student volunteer, is first of all given a pamphlet describing the history and uses of hypnotism. The subject is seen

at least twice before the experiment, and each time, after swallowing a stomach tube, hypnosis is induced and situations are suggested. If these preliminary tests are satisfactory, the full experiment is then performed. A continuous infusion of histamine is given as soon as hypnosis has been induced, and after 1 hr with the subject in a relaxed state, and when the gastric secretion is constant, the traumatic situation is suggested. After 1 hr in this phase, the subject is released, and kept in a state of rest for a further hour before ending the hypnotic period.

Emotional changes and pulse rate are recorded throughout the experiment, and gastric secretion is aspirated by continuous suction.

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## The measurement of total gastric blood flow and gastric secretion in the dog.

By J. D. CUMMING, A. L. HAIGH and E. H. L. HARRIES.
*Department of Physiology, University of Birmingham*

It is widely held that gastric secretion and gastric blood flow are directly related to each other. Harries (1956), however, suggests that this may not always be the case. The preparation described below has been used to obtain further evidence of the relationship between gastric secretion and blood flow. It involves the collection of the total venous outflow from the stomach, and the collection and estimation of the gastric secretion.

After induction with sodium thiopentone, anaesthesia is maintained through a tracheotomy opening with an ether oxygen mixture delivered from a Boyle's machine.

The abdomen is opened from xiphisternum to symphisis pubis through the linea alba. A double tape ligature is passed around the first part of the duodenum which is then divided. A similar procedure is adopted at the recto-sigmoid junction.

The whole of the gut is carefully lifted and its large vascular pedicle tied by means of a transfixing ligature. A large pair of Kelly's forceps is now put on above the pedicle tie for safety, and the vessels are divided to complete the evisceration. The spleen is next removed. A stomach tube is

then passed through the mouth and is used to guide the operator as a tape is put around the oesophagus above the cardia. This tape ties the tube firmly in position and occludes the oesophageal blood vessels.

The stomach is next turned back and the hepatic portal vein, superior mesenteric vein and splenic vein identified and cleaned by careful dissection. Thread ligatures are passed around them in such a way that with the portal vein tied a cannula placed in the superior mesenteric vein will collect the venous effluent from the stomach. If necessary a further tape is tied around the duodenum to include the pancreas and reduce the collection of drainage from this organ to a minimum.

One carotid artery and both femoral veins are prepared for cannulation.

The animal is given an hour's respite and then heparinized (50 mg). A carotid cannula is used to obtain a blood-pressure record. One femoral vein cannula is used to replace the venous blood collected from the superior mesenteric vein. The other one carries a fine length of nylon catheter connected to a constant rate infusion apparatus by means of which the effect of drugs upon both gastric secretion and stomach blood flow can be studied.

The stomach tube is connected to a constant-suction apparatus.

Blood flow is measured with a photo-electric drop counter and recorded on a kymograph by a Thorp impulse counter.

The effluent is returned to the animal at approximately this same rate through a warmed polythene coil and is impelled by a pump.

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**Qualitative assessment of variations in gingival blood flow in the rabbit.** By R. M. BROWNE. *Department of Dental Surgery, University of Birmingham*

The measurement of the temperature of the gingiva has been used as an index of rapid changes in the blood flow through it. The determinations were made with a simple thermocouple system.

The thermocouples were constructed from copper and constantan wires of a diameter of 0·019 cm (36 S.W.G.). The thermojunctions were soldered with a copper-zinc-silver alloy which contained no lead. The wires were enamelled and enclosed in a double-bore plastic sheath.

The thermojunctions were connected by the single constantan wire which was approximately 3 ft. (91 cm) long. Two copper wires led from the thermo-junctions to the recording instrument. These were also approximately 3 ft. in length. The wires being enclosed in double-bore tubing,

the entire wiring system consisted of a compact Y-shaped unit, each arm of the Y being approximately 18 in. long.

The variations in potential difference in the circuit were recorded with a Cambridge spot galvanometer of low resistance (approx. 25 Ω). The voltage produced by these thermocouples is 40 $\mu$V/° C temperature difference. The circuit was such that a full-scale deflexion was produced by approximately 350 $\mu$V, representing a range of about 9° C.

The reference thermocouple was placed in cedar-wood oil over ether which was boiling into a reflux condenser. Heat was provided by an electric light bulb. The temperature of the reference thermocouple was therefore maintained at 34–35° C, its exact value depending on the barometric pressure. With the galvanometer zeroed in the centre of the scale, a range of something like 31·0–40·0° C is obtained. The exact value of this scale was established by calibrating it with the feeler thermocouple adjacent to the bulb of a mercury thermometer and immersed in a water-bath: the calibration was made on each occasion that the apparatus was used and the linearity of the resulting graph established the integrity of the circuit.

Rabbits were anaesthetized with ether by the open drop method. Tracheotomy was performed and a set of respiratory valves attached to the tracheal cannula. Air breathed by the rabbits was then drawn over ether in a jar. The blood pressure was recorded by a mercury manometer from the femoral artery. The feeler thermocouple was passed down the gingival crevice, between the incisor tooth and its surrounding soft tissue until resistance to minimal pressure was encountered. It was maintained firmly in place by a supporting splint which was cemented to the tooth. This carried a small tube which was parallel with the long axis of the tooth and through which the plastic sheath just passed. When the thermocouple was correctly in place the plastic sheath was cemented into its supporting tube. The dimensions of the splint were such that the lips were able to lie *in situ* with a minimum of distortion.

By this method temperature variations in the gingivae in response to various stimuli were studied.

**A method for recording the electrical activity of the brain in the conscious rhesus monkey.** By P. B. Bradley and A. N. Nicholson. *M.R.C. Neuropharmacology Research Group, The Medical School, Birmingham* 15

**A new apparatus for human stereotactic surgery in the treatment of involuntary movement, epilepsy and intractable pain.** By B. Hughes. *Department of Neurosurgery, Queen Elizabeth Hospital, Birmingham* 15

**Macrophages in dental tissues of young rats.** By M. N. NAYLOR. *Department of Dentistry, Guy's Hospital, London, S.E. 1*

**Model heart.** By R. LIGHTWOOD and E. J. HAMLEY. *Department of Physiology, University of Birmingham*

**Equipment for micro-electrode study of normal cortex.** By I. C. WHITFIELD. *Department of Physiology, University of Birmingham*

**Binding of $^{14}$C–J 11 by isolated tissue.** By J. D. P. GRAHAM. *Department of Pharmacology, Welsh National School of Medicine, Cardiff*

Fifteen strips from the uteri of five pregnant rabbits were mounted in 10 ml. baths and the response to adrenaline 0·5 $\mu$g/ml. demonstrated. The dibenamine-like compound *N*-ethyl-*N'*-naphthylmethyl-2-bromoethylamine (J 11 of Graham & Lewis 1954), labelled with $^{14}$C on the methylene of the aromatic group to a specific activity of 4 $\mu$c/mg was added in a concentration of 1 $\mu$g/ml. and left for 30 min. At intervals up to 18 hr the response to adrenaline was tested, fluid being changed each time. The total specific activity in the washings and tissues was determined by a method involving the use of a liquid phosphor. In all cases the response to adrenaline was absent, and at 18 hr the tissue contained $7 \pm 2{\cdot}3\,\%$ of the total $^{14}$C-J 11 added. In twenty trials where the same amount of the labelled compound was added to a 50 ml. flask and to the baths $95 \pm 5{\cdot}4\,\%$ of the control activity was recovered. All radioactive material is cleared from a bath in three washes. Twenty strips were taken from seven uteri, ten put in baths containing 10 ml. of Krebs's solution and 10 in M/4 solution of $Na_2S_2O_3$ in Krebs's fluid. After 30 min all tissues were tested for response to adrenaline, removed, washed, weighed, homogenized and the washings and tissues counted in triplicate. The fluids containing thiosulphate had greater activity than the fluids without ($P < 0{\cdot}001$); the tissues exposed to thiosulphate contained less activity than those not exposed to it by some 50 % ($P < 0{\cdot}001$); the totals in the baths and tissues did not differ by more than 3 %; the tissues were no longer blocked to adrenaline in the characteristic non-competitive way by the added J 11. 10 $\mu$g (0·4 $\mu$c) $^{14}$C-J 11 was added to each of twenty-one collodion bags, containing 1·5 g homogenized rabbit uterus or liver, water, or 1·5 ml. plasma in 5 ml. Locke's solution and dialysed at 37° C in 25 ml. Locke's solution. Counts in triplicate from outside were done at intervals for 24 hr. The uterus and plasma bind the drug equally (about 30 %) at that time, the liver about 50 %. 100 $\mu$g compound was mixed with 0·9 ml. plasma, serum or Locke's solution and run for 16 hr at 150 V, 8 mA,

pH 8·6, on horizontal paper electrophoresis. Staining the protein and comparing it with autoradiographs showed that all the $^{14}C$-activity travelled with albumin.

The evidence suggests that dibenamine-like compounds are firmly attached to large protein molecules while activity persists.

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**Liberation of pyrogen by rabbit leucocytes.** By K. E. COOPER, W. I. CRANSTON and J. H. FESSLER. *M.R.C. Group for Research on Body Temperature Regulation, Department of the Regius Professor of Medicine, The Radcliffe Infirmary, Oxford*

Bennett & Beeson (1953) caused fevers in rabbits by injecting ground-up leucocytes from other rabbits. According to one hypothesis (Wood, 1958) leucocytes release a fever-producing material, called endogenous pyrogen, during fever. We find that, although active endogenous pyrogen is not present in leucocytes, it is made on incubation in normal saline.

Rabbit leucocytes were collected at 4° C from blood or from peritoneal exudates induced by sterile 3% starch solutions, and washed three times in 0·9% NaCl solution. Leucocyte preparations ($2 \times 10^7$ cells/kg) were injected intravenously into other rabbits and the areas of their temperature–time curves were used as fever indices (Wendt, Snell, Goodale & Cranston, 1956). The mean fever index of fifty uninjected rabbits was 0·25, S.E. 0·07,° C.hr. Cells incubated at 37° C for 1 hr caused fever (fever index 1·25, S.E. 0·10,° C.hr) while cells kept at 4° C were not pyrogenic (fever index 0·49, S.E. 0·17,° C.hr).

Ultrasonic disintegration of leucocytes at 4° C failed to release any pyrogen. This treatment did not affect the pyrogenicity of cells first incubated at 37° C. The formation of pyrogen was greatest after 1–6 hr incubation. It was abolished if the cells were first heated to 56° C for 30 min, or repeatedly frozen and thawed; these treatments did not affect the action of previously formed pyrogen.

Because rabbits are chronically infected with *Eimeria stiedae* (Macdonald, 1957), some were treated with nitrofurazone (5-nitro-2-furaldehyde semicarbazone). Leucocytes from such rabbits produced significantly less pyrogen, 0·37, S.E. 0·07,° C.hr, on incubation at 37° C. Nitrofurazone added *in vitro* before incubation to buffered cells from untreated animals also affects the formation of pyrogen. Neither intact nor ultrasonically disintegrated *Eimeria* spores caused a fever on intravenous injection.

Thus active pyrogenic material is not normally present in rabbit leucocytes, though it can be formed on incubation *in vitro*. Previous treatment of donor rabbits with nitrofurazone interferes with this process. While the pyrogenic material formed on incubation could be due to microbial contamination, bacteriological tests have been consistently negative and the most likely contaminant, *Eimeria stiedae*, is not pyrogenic when injected intravenously.

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**The calorigenic action of noradrenaline in the new-born kitten: its inhibition by hypoxia.** By R. E. MOORE and MARY C. UNDERWOOD. *Department of Physiology, Royal Free Hospital School of Medicine, London, W.C.* 1

It was recently reported (Moore & Underwood, 1960) that noradrenaline in doses of 400 $\mu$g/kg injected subcutaneously into new-born kittens at 35° C was calorigenic, whereas adrenaline was not.

In view of the effect of hypoxia in the new-born kitten in reducing heat production (Hill, 1959; Moore, 1959) it was of interest to determine the effect of hypoxia on the noradrenaline response. We therefore subjected unanaesthetized kittens at 35° C to various degrees of hypoxia before and after a single injection of L-noradrenaline (400 $\mu$g/kg). Oxygen consumption, rectal and skin temperatures were measured as previously described. It was observed that hypoxia consistently inhibited the noradrenaline stimulus to heat production in seventeen experiments on kittens between the ages of 12 hr and 20 days. Control experiments were carried out on all occasions with litter-mates, in all of which noradrenaline produced a marked rise in heat production.

It did not matter at which stage hypoxia was induced. If this was during the course of a noradrenaline response, then there was an immediate fall in heat production, if before, then either no response or a smaller response was observed. The degree of hypoxia influenced the result, inhibition beginning at an inspired $pO_2$ of between 100 and 120 mm Hg, and being complete at between $pO_2$ 70 and 80 mm Hg. If the animal was held in hypoxia until the expected duration of the noradrenaline response was over, then re-oxygenation produced no increase in heat production. Re-oxygenation before this restored the level of heat production to a level comparable with that of the control animal at the same time after injection of noradrenaline.

In other words, hypoxia did not simply delay the response, but appeared to suppress it.

In view of this finding it would be reasonable to interpret the hypoxic depression of heat production in new-born kittens as, in part at least, a depression of an existing noradrenaline calorigenic stimulus. This would explain why hypoxia is more effective a depressant at lower, than at higher environmental temperatures.

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**Initiation of cutaneous sensory nerve impulses.** By R. W. MURRAY. *Department of Zoology and Comparative Physiology, University of Birmingham*

The impulse discharge from single cutaneous fibres in elasmobranch fish can be recorded without dissection by a wire electrode resting lightly on the surface of the skin and an indifferent electrode elsewhere on the body. Mechanical stimulation with or alongside the wire electrode results in a brief burst of impulses completely adapting in a few seconds.

The individual action potentials are diphasic and each phase is of longer duration (1–8 msec) than when recorded conventionally from the same nerves farther from the skin. The first phase is always positive at the electrode at the stimulated site. The shape and polarity of the potentials resulting from this mechanical stimulation are the same as those resulting from antidromic, electrical stimulation of the nerve more proximally. The latency of impulse initiation with mechanical stimulation is longer the weaker the stimulus, with an observed limit of 30 msec in one preparation at threshold, falling to about 4 msec with strong stimulation.

The nerve fibres run in small bundles through channels in the compact fibrous layer of the dermis, lose their myelination and end in fine branches beneath and probably in the epidermis. It is suggested that this fibrous layer forms a high-resistance region effectively separating the two electrodes in the same way as an air gap separates the two pools of saline in an isolated fibre experiment. Recording of impulses is only possible while they pass through this region, the potentials being short-circuited by the tissue fluids when they are elsewhere. The positive phase of the diphasic potential thus represents activity of the nerve at the end of the channel through the fibrous layer farther from the surface, and the negative phase activity at the surface end.

It is suggested that the effect of the mechanical stimulus on the nerve endings must be transmitted by a means not experimentally detectable to a point on the stem axon lying on the far side of the fibrous layer where a propagated impulse is initiated. From this point the impulse must travel in both directions along the nerve, not only onwards to the C.N.S. but also back towards the skin, and it is the latter antidromically propagating action potential which is recorded, even with mechanical stimulation. The way in which the presumed depolarization of the non-myelinated terminal regions of the nerve could influence the stem region where impulses are initiated may be either simple electrotonic spread, in which case the long latencies are to be explained in terms of a long 'response time' (Hodgkin, 1948), or else a kind of slow, graded and possibly decremental conduction analogous to that found in dendrites (see Purpura, 1959).

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**Reflex activation of vasodilator nerve fibres to skeletal muscle in decerebrate and intact cats.** By V. C. ABRAHAMS, S. M. HILTON and A. ZBROZYNA*. *The National Institute for Medical Research, Mill Hill, London, N.W. 7*

Certain regions of the hypothalamus and mesencephalon appear to act as reflex centres for the defence reaction (Abrahams, Hilton & Zbrozyna, 1959; Abrahams, Hilton & Malcolm, 1959). Part of this reaction is a cardiovascular response which includes active muscle vasodilatation.

Active muscle vasodilatation has now been obtained as a reflex response in high decerebrate cats, in which the hypothalamus and mesencephalic structures have been spared. When the ether has worn off, electrical stimulation of the skin produces most of the signs of the defence reaction including the features observed in similar experiments by Woodworth & Sherrington (1904) and termed the pseudaffective reflex.

In our decerebrate cats venous outflow was recorded from an innervated gastrocnemius muscle perfused by a heparinized donor cat. Movements of the perfused muscle were prevented by section of the spinal cord between L5 and L6. It was found that the reflex response to skin stimulation included a large and prolonged increase in muscle blood flow due to activation of cholinergic vasodilator nerve fibres.

When decerebration removed a substantial part of the hypothalamus

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the skin stimulation no longer produced muscle vasodilatation but still elicited many features of the behavioural response.

If the defence reaction is produced in the intact non-anaesthetized cat by electrical stimulation of the hypothalamus, the response is graded according to the strength of stimulation. Threshold stimulation does not produce fight or flight. It produces alerting, but already with pronounced muscle vasodilatation, as shown when muscle blood flow is registered using the venous temperature technique (Abrahams, 1959). Confirmation that this vasodilatation, like that in the decerebrate cat, is due to activation of cholinergic vasodilator fibres was obtained by showing that it is reduced or abolished by atropine.

These same behavioural and cardiovascular responses are obtained reflexly in the intact cat on nociceptive stimulation such as electric shocks to the foot pads. Any stimulus which produces the alerting reaction also elicits the active muscle vasodilatation. For instance, a loud note sustained for 10 sec usually produces these responses at least at the first trial. On repetition of the note, however, the responses usually diminish and are absent by the 5th trial. In a few cats this habituation to repeated stimulation was not seen.

When an auditory stimulus does not itself produce responses it is possible to show that a conditioned vasodilator response can be developed to such a stimulus, by combining it with nociceptive stimulation 4 or 5 times.

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**The chemical nature of the menstrual stimulant.** By R. F. C. Brown, H. J. Clitheroe and V. R. Pickles. *M.R.C. Department of Experimental Medicine, University of Cambridge*

**Changes in oxygen consumption and cytochrome C in the diaphragm of acclimatized rats.** By Marjorie W. Duckworth. *Department of Physiology, Royal Free Hospital School of Medicine, London, W.C.* 1

Previous investigations into the mechanisms involved in the physiological adaptation to chronic hypoxia have established respiratory and circulatory changes. In spite of these adaptations it is probable that the tissue oxygen pressure is still below normal in the fully acclimatized state;

whether such a low pressure of oxygen in the tissues produces any adaptation of the cellular metabolism has prompted investigations into tissue oxygen consumption.

In the present work oxygen uptakes of the diaphragm muscle have been measured by the Warburg technique; the tissues were taken from rats kept under normal atmospheric conditions and from rats acclimatized for several months to approximately 10% oxygen in inspired air. Results showed that the $q_{O_2}$ ($\mu$l. $O_2$/mg wet wt. tissue/hr) was reduced in the diaphragm from acclimatized animals, particularly at low *in vitro* oxygen pressures.

There was no significant difference in the water content of the tissues from the two groups of animals. The levels of cytochrome C in the diaphragm muscle were significantly lower in the acclimatized rats.

**Identification of a new neurohypophysial hormone.** By H. Heller and B. T. Pickering. *Department of Pharmacology, University of Bristol*

Neurohypophysial extracts from cold-blooded vertebrates and birds affect the water balance of frogs more markedly than extracts of mammalian pituitaries (Heller, 1941; Sawyer, 1957). This led to the hypothesis that they contain a peptide hormone (provisionally called 'water balance principle') which is neither oxytocin nor vasopressin (Heller, 1941). Recently further pharmacological and chromatographic evidence has been advanced for the occurrence of such a hormone in the pituitaries of teleosts, amphibians, reptiles and birds (Pickering & Heller, 1959; Sawyer, Munsick & van Dyke, 1959). Attempts have now been made to identify this peptide.

A procedure for the purification of oxytocin and vasopressin (Acher, Light & du Vigneaud, 1958), entailing ion-exchange chromatography, was applied to extracts of lyophilized fish (pollock) pituitaries. The active material thus obtained was further purified by paper chromatography in butanol–acetic acid–water.

Sawyer *et al.* (1959) found that extracts of the pituitaries of several lower vertebrates, including teleosts, had the pharmacological properties of arginine vasotocin, a synthetic vasopressin analogue prepared by Katsoyannis & du Vigneaud (1958). The purified pollock pituitary peptide was, therefore, compared with arginine vasotocin (kindly supplied by Professor du Vigneaud).

Paper chromatograms of vasotocin and the pollock peptide, side by side, were developed with three solvent systems. In each case the pollock peptide moved the same distance as vasotocin. The $R_F$ values in each

solvent were as follows: butanol–acetic acid–water, 4:1:5 (descending), 0·31; ammonium sulphate–cellosolve–water, 20:24:56 by weight (descending), 0·75; ethyl acetate–acetic acid–formic acid–water, 18:3:1:4 (ascending), 0·13.

A sample of the purified peptide was hydrolysed with 6N-HCl at 110° C for 48 hr and the hydrolysate subjected to two-dimensional paper chromatography in (*a*) butanol–acetic acid–water and (*b*) α-picoline–water. A mixture of the hydrochlorides of the amino acids present in vasotocin was chromatographed simultaneously. Both chromatograms were stained first with ninhydrin and then by the technique of Reindel & Hoppe (1954). All the spots visible on the chromatogram of the mixture also appeared on that of the peptide hydrolysate, but the latter showed two additional spots, one corresponding to alanine and another, not revealed by ninhydrin, probably due to a peptide fragment.

These results, together with the pharmacological evidence, lead us to believe that the peptide isolated from pollock pituitaries is identical with arginine vasotocin, which has the structure

$$\text{CyS.Tyr.Ileu.Glu}(NH_2)\text{.Asp}(NH_2)\text{.CyS.Pro.Arg.Gly}(NH_2)$$

We are much indebted to Dr Grace Pickford and Professor A. E. Wilhelmi for the supply of the pollock pituitary powder.

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**The effect of calcium on rat diaphragm muscle fibres.** By D. H. Paul. *Department of Zoology and Comparative Physiology, University of Birmingham*

Calcium has previously been observed to produce a depression of the twitch height of an indirectly stimulated rat phrenic-nerve diaphragm (Paul, 1957), and the action was ascribed to a direct effect on the excitability of the muscle fibres. This type of depression can be obtained equally well on a curarized preparation, and can be recorded both isotonically, and isometrically. A 4 mM increase in $CaCl_2$ concentration produces a marked depression of twitch height or tension within a few seconds. This effect is not osmotic and cannot be duplicated by increasing the NaCl concentration.

Measurements of membrane potentials of muscle fibres have also been

made with glass micropipettes filled with 2·5 M-KCl. Increasing the $CaCl_2$ concentration by 10 mM resulted in a rise of membrane potential from 83·0 mV ± 1·4 (S.E. of 12) to 91·0 mV ± 1·2 (S.E. of 8; $P < 0.01$ by $t$ test). Similar results have been reported for both frog and rat muscle fibres (Jenerick & Gerard, 1953; Grossweiler, Kipfer, Poretti & Rummel, 1954).

No effect could be demonstrated on the tissue contents of $Na^+$ and $K^+$ in muscles soaked in Krebs's solution with high $CaCl_2$ concentration. In normal Krebs's solution (2·6 mM-$CaCl_2$) after 1·5 hr soaking the $Na^+$ content was 69·2 m-equiv/kg wet tissue ± 3·5 (S.E. of 8) and the $K^+$ content was 52·0 m-equiv/kg wet tissue ± 1·9 (S.E. of 8). Corresponding figures for muscles soaked in solutions with 3 and 5 times normal $CaCl_2$ were $Na^+$, 71·8 ± 3·9 (S.E. of 7) and 69·6 ± 6·0 (S.E. of 5); $K^+$, 50·8 ± 2·6 (S.E. of 7) and 55·0 ± 3·1 (S.E. of 5). In neither case is the $Na^+$ or $K^+$ content of a tissue soaked in a solution with high $CaCl_2$ concentration significantly different from the value for a tissue soaked in normal Krebs's solution.

These results are in agreement with the many observations that high concentrations of $Ca^{2+}$ reduce the excitability of excitable tissues in general by causing hyperpolarization.

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**Equilibration between water vapour and human skin.** By P. DIRNHUBER and R. T. TREGEAR. *Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wiltshire*

Buettner (1959) suggested that water is reabsorbed actively by the epidermis. If so, free water should never appear on the surface of non-sweating skin and the aqueous vapour pressure (V.P.) at equilibrium with the skin surface should always be lower than the S.V.P. of water at the surface temperature ($p_s$).

Insensible water loss from the human back was studied by measuring the changes in V.P. within a cell (2 cm × 30 $cm^2$) pressed on to the skin. The cell was kept at a constant temperature ½–2° C above that of the skin within it (37–39° C) to prevent water condensing on the walls, and the V.P. was measured with a dew-point hygrometer; ten tests were carried out. The subjects were prone, clothed, in a room at 20–23° C. As it is unusual to obtain local sweating at skin temperatures under 40° C (Randall, 1947) sweating was not to be expected and none was detectable within the cell; moreover, atropine electrophoresis (0·3 coulombs/$cm^2$) had no effect on the

equilibrium V.P. obtained, although it greatly reduced the local sweat response to exercise.

After the cell was sealed the V.P. rose exponentially with time; the calculated rate of water loss at a V.P. of 15 mm Hg (23° C at 75 % r.h.) was 5 $\mu$g/cm$^2$/min, or approx. 0·2 $\mu$g/cm$^2$/min/mm Hg difference from $p_s$. This is somewhat lower than has been previously reported (Pinson, 1942) possibly because the air in our cell was unstirred. The exponential rise of V.P. was nearly complete in 20 min, but instead of reaching a steady level it continued to increase slowly (approx. 0·05 mm Hg/min). After 100 min the pressure was, on average, 5 mm Hg below $p_s$, and just perceptibly rising. In three experiments, continued longer, dew was allowed to condense on the hygrometer for 20 min and was then dispelled. This raised the V.P. to within 2 mm of $p_s$, from which level it fell extremely slowly (approx. 0·01 mm Hg/min) during the following 150 min, suggesting that equilibrium occurs between 3 and 5 mm Hg below $p_s$.

When a small free water surface was introduced into the cell the full value of $p_s$ was quickly reached so that very little, if any, free water can have been present on the skin in our experiments and the keratin cannot have been completely saturated with water. Whether this was due to incomplete equilibration in the time available or to an active reabsorption is uncertain. If the latter, the rate of reabsorption was probably not greater than 1 $\mu$g/cm$^2$/min, for the V.P. depression was 3–5 mm Hg and the water loss 0·2 $\mu$g/cm$^2$/min/mm Hg below $p_s$.

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**Potassium salts and the resting potentials observed with micropipette electrodes in slices of mammalian cerebral cortex *in vitro*.** By H. H. HILLMAN and H. MCILWAIN. *Department of Biochemistry, Institute of Psychiatry, London, S.E.* 5

The slices, 300 $\mu$ thick, were incubated at 37° C at the surface of oxygenated glucose salines buffered with bicarbonate–$CO_2$, and maintained resting potentials observed (Li & McIlwain, 1957). In nine recent experiments with slices from the parietal cortex of the guinea-pig and the frontal lobe of man, and taking the maximum voltage recorded in each penetration, 269 penetrations showed resting potentials averaging $58 \pm 12$ mV (S.D.). Potentials were normally observed for periods of some seconds, and in some cases were stable for several minutes; any lasting less than 0·1 sec were not taken into account. Tissues from the rat gave similar values.

In tissue incubated in media which initially contained no potassium salts, no resting potentials could be recorded. Subsequent addition of KCl to 5 mM resulted in the appearance of a few potentials 30–40 mV in amplitude. In tissue incubated in media with less than 5 mM-$K^+$, resting potentials were lower than in tissues from media 5 mM in $K^+$, and higher potentials were recorded on adding KCl to give 5 mM-$K^+$. Resting potentials decreased with increase in $K^+$ between 5 and 15 mM, usually being absent at $K^+$ concentrations of 15–20 mM. After such depolarization, change of media to one with 5 mM-$K^+$ restored potentials.

The additional presence of glutamate ions in glucose salines has been reported to raise the $K^+$ associated with a given weight of cerebral tissue (Terner, Eggleston & Krebs, 1950; Pappius & Elliott, 1956). In the present experiments addition of Na L-glutamate to 20 mM resulted in a significant fall in resting potential; in four experiments totalling 105 readings, values of $60 \pm 17$ mV (mean and S.D.) were recorded during the control period; these became $44 \pm 20$ with glutamate ($P < 0{\cdot}001$) and rose to $51 \pm 19$ ($P = 0{\cdot}01$) on changing the solution back to one without glutamate. The fall might be related to the action of glutamate in depleting the tissue phosphocreatine (McIlwain, 1952).

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**Gangliosides and basic proteins which condition the response of cerebral tissues to electrical pulses.** By H. McIlwain. *Department of Biochemistry, Institute of Psychiatry, Maudsley Hospital, London, S.E.* 5

Mutual effects of gangliosides and basic proteins on the response, shown first by examining an unresponsive state induced by keeping cerebral tissues in cold media (McIlwain, 1959, 1960), have now been studied in more detail by adding the individual materials to normal cerebral tissue. Respiration and glycolysis, and respiratory and glycolytic response to electrical pulses, were measured while the tissues were in saline–glucose mixtures with the added materials. Gangliosides, which occur almost exclusively in neural tissues (Long & Staples, 1959) and are large molecules of unusual oil–water solubility, were prepared according to the method of Folch, Lees & Sloane-Stanley (1957).

The basic proteins were potent inhibitors of response to pulses. Protamine specimens were found to inhibit by 50% when added at 40 $\mu$g/ml. or about 0·9 $\mu$mole/g tissue; a synthetic poly-L-lysine was similarly active at 50 $\mu$g/ml. or 1 $\mu$mole/g tissue; and histone preparations at 80 $\mu$g/ml. or about 0·4–0·6 $\mu$mole/g tissue. After inhibition by incubation with the protamine, response was not restored by repeatedly washing the tissue nor by incubation in media lacking protamine. Response was restored by incubation with media to which ganglioside preparations had been added at 130 $\mu$g/ml. or about 0·7 $\mu$M.

Neuraminic acid, which gives the gangliosides their acidic character, occurs in the cerebral cortex at 1·7 $\mu$mole/g tissue (Long & Staples 1959); 50% inhibition by the basic proteins and polypeptides was thus caused by quantities of them which were 30–50% of the molar equivalent of the neuraminic acid of the tissue specimen on which they acted. Combination may be pictured to be analogous to that of the electron micrographs with which Katchalsky, Danon, Nevo & de Vries (1959) have illustrated the combination of the basic polypeptides with erythrocytes. Neuraminic acid of large molecules also contributes the major acidic groups to the erythrocyte membrane (Gottschalk & Klenk: Symposium, 1958). In cerebral tissues an analogous action at membranes could be understood to impede ion movement associated with the tissue's response. A normal role of the groups with which the protamine interacts is adumbrated in the finding that inhibition by protamine is markedly dependent on the calcium content of the medium to which it is added. Inhibition of response by 0·1 mg of a protamine/ml. fell from about 95% in media 0·54 or 2·7 mM in $Ca^{2+}$, to < 10% in media with 27 mM $Ca^{2+}$.

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**Adenosinetriphosphatase activity and ion movements.** By E. T. Dunham and I. M. Glynn. *Physiological Laboratory, University of Cambridge*

The mammalian red cell membrane uses glycolytic energy to pump K into the cell and Na out of it. The present experiments were designed to see whether the ATP-ase activity known to be associated with the cell mem-

brane (Clarkson & Maizels, 1952; Caffrey, Tremblay, Gabrio & Huennekens, 1956; Herbert, 1956) is connected with these ion movements.

Washed human red-cell ghosts were incubated at 37° C with salt solutions containing ATP, and the rate of splitting was followed by measurement of the inorganic phosphate formed. The effects of pH, ATP concentration, Mg, Ca, Na, K, and strophanthin (ouabain) were studied.

In the absence of divalent cations the ghosts showed almost no ATP-ase activity. Low concentrations of Mg had a powerful activating effect. Ca ions alone caused only slight activation but, in the presence of Mg, Ca caused very marked activation at low concentrations and inhibition at higher concentrations. The level at which inhibition occurred depended on the Ca: Mg ratio, not on the absolute level of Ca.

The effects of Mg and ATP concentration on activity cannot be explained simply in terms of the concentration of Mg–ATP complex in the solution, and it seems that Mg must affect the enzyme or enzymes directly.

In the presence of Mg and absence of Ca activity was much increased if both Na and K were added, though either ion alone had no effect. The size of the increase depended on the Mg level. The relationship between the increase in activity and the relative amounts of Na and K added is complicated, as the optimal concentration of each depended on the concentration of the other. Strophanthin at a concentration of $10^{-5}$ M, which is known to inhibit Na and K transport (Schatzmann, 1953), completely prevented the activating effect of Na + K on ATP-ase activity. A similar result was reported recently by Post (1959). At much lower concentrations of strophanthin ($6{\cdot}8 \times 10^{-8}$ M) inhibition of the extra activity was marked at low K concentrations but became progressively less as the K concentration was raised. This is significant because the inhibition of K influx in intact red cells by low concentrations of cardiac glycosides is similarly reversed by raising the external K concentration (Glynn, 1957). Experiments with certain modified cardiac glycosides showed that modifications which are known to reduce the inhibitory effect on the pump (Glynn, 1957) also reduced the inhibitory effect on the $Na^+K$ activated ATP-ase.

The results suggest that there is an intimate connexion between active ion movements and the splitting of ATP in the membrane.

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**Responses of single neurones in nucleus gracilis of the cat to lemniscal and cerebellar stimulation.** By G. GORDON and W. A. SEED*. *Laboratory of Physiology, University of Oxford*

N. gracilis is functionally differentiated, in that cells in its middle (3 mm long) section have small skin receptive areas while those in the caudal and more particularly in the rostral (4 mm long) section have large areas; and mutual inhibition is prominent among cells of the middle section (Gordon & Paine, 1959). We have investigated this organization further by firing impulses into the nucleus, under pentobarbitone sodium anaesthesia, either from the contralateral medial lemniscus in the caudal thalamus or from the cortex of the ipsilateral anterior lobe of the cerebellum.

Responses of those cells whose receptive areas included the hind foot fell into two categories:

(*a*) Cells responding to single shocks with one spike at constant threshold and with short fixed latency (lemniscus 1–3 msec; cerebellum 1–1·9 msec). At twice threshold an early second shock was effective after 0·4–0·9 msec, and brief tetani of 500–1350/sec were followed. All figures represent ranges observed in the cell population studied. We consider such responses to be *antidromic*.

(*b*) Cells responding to single shocks with one or more spikes, with variable threshold and long variable latency (3·5 msec upwards); and responding to second shocks after 1·5–4·5 msec. We have called these *trans-synaptic responses*.

Cells responding *antidromically* to lemniscal stimulation were distributed throughout the nucleus. In the middle section 84% (of 80) cells were fired—in one experiment all cells found there (15) were fired. Percentages in the other sections were lower (rostral 73%; caudal 58%). Antidromic responses to cerebellar stimulation were investigated in a few experiments: eleven such cells were found in the rostral section and none elsewhere, and their receptive areas were among the largest even in this section (mean 60 $cm^2$).

Cells responding *trans-synaptically* were almost confined to the rostral section (11 out of 12 cells studied, in a limited investigation), conditions being such that their presence elsewhere would have been noticed. All were fired from the lemniscal region at thresholds comparable with those for antidromic responses. Their skin areas were normal for the rostral section (mean 15 $cm^2$).

Probably almost all the cells in the middle section of the nucleus project directly to the thalamus through the lemniscus, and there are few if any interneurones. Many cells in the rostral and caudal sections also project in this way; though some in the rostral section project to the cerebellum.

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Several explanations are possible for the trans-synaptic effects. It seems likely that they result from antidromic excitation of axons having collaterals ending directly or through interneurones on the cells studied. One possibility is that cells in the rostral section owe their wider receptive areas to convergence of collaterals from cells with smaller areas lying more caudal.

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**The effect of temperature and pH on the facilitated transfer of glucose across the human erythrocyte membrane.** By A. K. SEN and W. F. WIDDAS. *Department of Physiology, King's College, London, W.C.2*

By following the volume changes of red cells during glucose loss into outside media containing low concentrations of glucose it is possible to determine: (i) the half-saturation constant (Sen & Widdas 1960), and (ii) the maximum rate of the facilitated transfer system for sugars.

This technique has been used to study the effects of pH at 37° C and of temperature change at pH 7·4.

The effect of pH was studied over the range pH 5·4–8·4, but the half-saturation constant ($\emptyset_g$) only varied from about 3·4 at pH 5·4 to 4·1 at pH 8·4. The change was just statistically significant ($P < 0{\cdot}05$). The maximum rate of exit showed greater variations over this pH range which were statistically significant ($P < 0{\cdot}001$) and could be represented by the regression equation.

Maximum rate ($K$) = 0·17 (pH − 0·87) iso-quantities/min.

The change with temperature was more striking. The half-saturation constant decreased at low temperatures and the variation between 7° C (0·6 mM) and 37° C (4·0 mM) was more than sixfold. It was found that the logarithm of the half-saturation constant varied linearly with the reciprocal of the absolute temperature and the slope of such a plot (which is negative) corresponds to an energy of 10,000 cal/mole being required for the dissociation of the glucose complex.

The maximum rate of transfer varied by a factor of 22 over the same temperature range and a plot of the logarithm of the rate was not linear with the reciprocal of the absolute temperature but was concave downwards. This type of plot has been discussed by Stearn (1949) in regard to successive reactions in enzyme studies.

These findings suggest that whereas the formation and dissociation of a complex between glucose and a membrane component could be a simple chemical process, passage of glucose (or the complex) through the membrane

does not depend simply on a single activation energy and could involve a series of steps.

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**The effect of cooling the legs on the rate of sweat production from the forearm, when the rest of the body surface is not exchanging heat.** By D. F. BREBNER and D. MCK. KERSLAKE. *R.A.F. Institute of Aviation Medicine, Farnborough, Hants.*

The subject was immersed in a stirred water-bath maintained at his mouth temperature. His head was covered by a box ventilated with saturated air at this temperature. Sweat was collected from a capsule covering 12 $cm^2$ of the inner surface of the right forearm. Dry air was passed through the capsule at 3 l./min, and the evaporated sweat collected over 4 min periods in weighed drying tubes. The legs and feet could be cooled by raising them out of the water, and the degree of cooling could be varied by changing the air movement over the exposed wet skin. The skin temperature of the legs and feet was measured by ten thermocouples tied on with thin elastic cord.

With the legs immersed, the sweat rate from the forearm was found to be linearly related to the mouth (and bath) temperature over the range 37–38° C. Cooling the legs produced a fall in sweat rate relative to that which would have been expected had the legs not been cooled. This reduction was proportional to the reduction in leg temperature, and there was no evidence that the change in posture was affecting the sweat rate.

A series of experiments was carried out to investigate the effect of occluding the circulation to the cooled legs. Each consisted of three observations of sweat rate with the legs immersed unoccluded, between which were interposed observations under two of the following conditions: legs cooled unoccluded, legs cooled occluded or legs occluded but not cooled. A block of six experiments in which each pair of these conditions was presented in each order was performed on each of three subjects. It was found that cooling the unoccluded legs always depressed the sweat rate, but that cooling the occluded legs did not depress it, i.e. the sweat rate was the same as if the legs had not been cooled. Occluding the circulation to the uncooled legs was without effect on the sweat rate.

The results can be explained by supposing that there are no cutaneous receptors controlling sweat rate, which depends on deep body temperature (Benzinger, 1959) and its rate of change. Alternatively, if there are cutaneous receptors, then either they fail rapidly after occlusion of the circulation and their output is zero when no heat is exchanged, or they are

sensitive to a temperature difference which approaches zero when the circulation is stopped, although heat is being lost. The receptors postulated by Bazett (1951) are consistent with the observations.

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**Pulmonary diffusing capacity on exercise in athletes and non-athletic subjects.** By R. G. BANNISTER*, J. E. COTES, R. S. JONES† and F. MEADE. *Pneumoconiosis Research Unit of the Medical Research Council, Llandough Hospital, Penarth, Glamorgan*

The attainment of a high oxygen intake requires a high diffusing capacity for oxygen ($D_{Lo_2}$) (Shepard 1958). High $D_{Lco}$ values have been obtained in fit subjects (Heinonen, Karvonen & Ruosteenoja, 1958); few measurements on athletes have so far been reported (e.g. Bates, Boucot & Dormer, 1955).

The present study concerns seven British middle-distance competitors in the 1958 Commonwealth Games at Cardiff and eleven control subjects; their mean ages, surface areas and functional residual capacities were 24·8 and 24·3 years, 1·78 and 1·85 $m^2$ and 4·03 and 4·19 l. respectively. $D_{Lco}$ was measured by a modification of the single breath method of Forster, Fowler, Bates & van Lingen (1954) in which allowance is made for the changes in CO concentration during the inspiration and expiration of the test gas (Jones & Meade, 1960). Values so obtained are unaffected by the length of time of breath-holding: in the present study times of 6–10 sec were employed.

For both groups of subjects $D_L$ during exercise increased by 1·82 ml./min/mm Hg/kcal/min increase in energy expenditure (Table 1) but, at the levels of energy expenditure studied, the values in the athletes were systematically higher ($P < 0{\cdot}05$). These results may reflect a larger area or increased permeability of the alveolar capillary membrane in the athletes without there necessarily being a greater volume of blood in their alveolar capillaries.

Measurements were made during near-maximal exercise in a few subjects. These confirm the finding of Ogilvie, Forster, Blakemore & Morton (1957) that $D_L$ obtained by the single breath method increases linearly with exercise energy expenditure. The results therefore resemble those obtained for $D_L$ by the steady state CO method rather than by the oxygen method which, when determined simultaneously, gives values for $D_L$ that

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rise to a plateau at a relatively low level of energy expenditure (Shepard *et al.* 1958).

TABLE 1. Energy expenditure ($E_{O_2}$ kcal/min) and single breath pulmonary diffusing capacity ($D_L$ ml./min/mm Hg) of subjects at rest and walking at 2½ and 4 miles/hr (4 and 6 km/hr) on a horizontal treadmill

| | Rest | | 2½ miles/hr | | 4 miles/hr | |
|---|---|---|---|---|---|---|
| Number | $E_{O_2}$ | $D_L$ | $E_{O_2}$ | $D_L$ | $E_{O_2}$ | $D_L$ |
| | | | Athletes | | | |
| 1 | 1·24 | 41·0 | 3·23 | 44·4 | 4·62 | 47·9 |
| 2 | 1·82 | 32·2 | 4·27 | 37·7 | 6·61 | 40·9 |
| 3 | 1·71 | 39·1 | 7·60 | 47·2 | 9·94 | 48·6 |
| 4 | 1·72 | 37·7 | 4·16 | 42·6 | 6·25 | 47·9 |
| 5 | 2·07 | 40·6 | 4·25 | 43·7 | 6·57 | 46·5 |
| 6 | 1·46 | 29·1 | 2·61 | 40·0 | 4·91 | 44·0 |
| 7 | 1·45 | 33·1 | 3·90 | 45·6 | 5·76 | 49·6 |
| Mean | 1·64 | 36·11 | 4·29 | 43·0 | 6·38 | 46·5 |
| | | | Non-athletes | | | |
| 11 | 1·45 | 36·3 | 4·60 | 39 4 | 7·19 | 42·1 |
| 12 | 1·57 | 34·3 | 5·01 | 39·7 | 7·11 | 43·2 |
| 13 | 1·37 | 32·2 | 4·59 | 41·7 | 8·41 | 44·7 |
| 14 | 1·48 | 36·3 | 4·78 | 43·6 | 8·20 | 48·3 |
| 15 | 1·85 | 31·3 | 4·68 | 41·5 | 8·05 | 44·4 |
| 16 | 1·56 | 29·6 | 5·37 | 35·7 | 6·43 | 36·7 |
| 17 | 1·52 | 31·4 | 4·91 | 35·0 | 8·01 | 43·7 |
| 18 | 1·20 | 27·2 | 3·75 | 31·9 | 5·36 | 35·0 |
| 19 | 1·95 | 25·1 | 4·32 | 32·5 | 6·26 | 34·5 |
| 20 | 1·52 | 30·0 | 4·14 | 36·9 | 6·09 | 38·7 |
| 21 | 1·26 | 30·3 | 4·21 | 34·5 | 7·48 | 38·0 |
| Mean | 1·52 | 31·3 | 4·58 | 37·5 | 7·15 | 40·8 |

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**The production of selective deafness by direct suggestion under hypnosis.** By S. BLACK and E. R. WIGAN. *Psychiatric Department, The West London Hospital, Hammersmith, London, W. 6, and The Engineering Research Department, British Broadcasting Corporation, Kingswood Warren, Tadworth, Surrey*

The auditory thresholds of six deep-trance hypnotic subjects with normal hearing were measured in the waking state and again after deafness to tones of specific frequency had been suggested under hypnosis.

The subject lay on a couch and wore high-fidelity headphones fitted with soft rubber earpads to minimize ambient noise, which was estimated at 30/40 Phons. The auditory thresholds were measured between 100 c/s and 10,000 c/s, and two methods were used for presenting the test tones in the headphones. In the first method tones of variable but precisely known pitch were produced as a series of pulses from a circuit fed by a Muirhead-Wigan decade audio-oscillator. By use of a variable octave filter any distortion due to the oscillator or the pulse generator was kept to less than 0·1 %. The intensity of the tone was controlled by the operator and different frequencies were presented at random. In the second method a Békésy audiometer provided a gliding tone, the frequency rising throughout the test at the rate of about half an octave per minute. In this method the intensity was controlled by the subject and the result recorded automatically. In all experiments, during the suggestion of selective deafness to the hypnotized subject, the specific frequency involved was presented as a constant tone at an intensity level of 80 db sound pressure level (S.P.L.), over a period limited to 20 sec.

It was found that hearing of tones at the specific frequencies tested between 250 and 1000 c/s, and at a maximum intensity of 100 db S.P.L. (the maximum considered safe for the subject at the duration used), could be inhibited by suggestion. In some subjects this inhibition tended to decay after periods varying from 10 to 30 min, while in others inhibition remained until removed by counter-suggestion. In certain subjects this selective deafness sometimes included not only the test frequency but also half and twice this frequency.

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**Effect of local cooling on glucose metabolism in the human forearm.** By ELLEN BROWN* and R. F. MOTTRAM. *M.R.C. Group for Research on Body Temperature Regulation, Department of the Regius Professor of Medicine, The Radcliffe Infirmary, Oxford*

It is known that in man and dog (Wynn, 1954, 1956), and in the rabbit (Bickford & Mottram, 1958, 1960), the disappearance from blood of parenterally administered glucose is depressed by hypothermia with anaesthesia. We therefore studied the effect of local cooling on the uptake of glucose by human forearm muscle. In twelve experiments on five healthy young men, forearm blood flow was measured by venous occlusion plethysmography at intervals of 10 min starting 50–138 min, and ending 95–216 min, after beginning a substantial breakfast. The water temperature of the

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plethysmograph was either neutral (34° C) or cool (18–20° C). Immersion at 18–20° C lowers deep temperatures of the forearm to 25–30° C (Clarke, Hellon & Lind, 1958). In the course of seven experiments the plethysmograph temperature was changed from neutral to cold, or vice versa, and an interval of at least 30 min was allowed for equilibration before resuming observations. Before and after each measurement of flow, blood was taken through an indwelling catheter from a deep vein of the same forearm and, simultaneously, from a wrist vein of the opposite (gloved) hand which remained continuously in a bath of water at the maximum tolerated temperature (45–47° C). The $O_2$ content of venous blood from the heated hand was 0·0–0·5 vol. % lower than that of blood taken simultaneously from an artery in three experiments. Glucose differences of an equivalent size cannot be precisely determined. The glucose concentration in this venous blood is therefore considered here as 'arterial'.

At 34° C, total flow averaged 7·5 ml./100 ml. forearm/min (3·8–16·6 ml.) and muscle flow, calculated from previously determined regression equations, averaged 4·8 ml. (range 4·1–7·9). At 18–20° C, total flow averaged 2·4 ml./100 ml. forearm/min (range 1·1–3·8) and muscle flow 3·6 (range 1·7–5·9). During cooling, muscle flow fell in five experiments and rose slightly in two.

The results of blood glucose determinations from all experiments were averaged for each 10 min interval of experimental time. Arterial concentration 50 min after breakfast averaged 150 mg %; it fell between 80 and 90 min to 90–100 mg % and thereafter remained steady. Glucose A–V difference at 34° C was 20–30 mg % throughout, except for a rise to 40 mg as the arterial level fell. Cooling was accompanied by a fall of A–V difference in four experiments, no change in one, and a rise in two; the average change was −4 mg %. Glucose uptake (muscle flow × A–V difference) at 34° C averaged 1·28 mg/100 ml. muscle/min (range 0·67–1·93) and at 18–20° C 0·88 (range 0·47–1·75). This change was statistically significant. Similar changes occurred in $O_2$ A–V difference and uptake on cooling. The ratio of glucose to $O_2$ A–V differences expressed as chemical equivalents was 3·0 at 34° C and 2·6 during cooling.

It is concluded that the effects of local cooling on blood flow and glucose uptake of forearm muscle, if considered representative of skeletal muscle as a whole, cannot account for all the disturbances of glucose metabolism previously found in anaesthetized animals subjected to general body cooling. However, the plan of the present experiments is different from those performed on the anaesthetized preparations.

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**Absorption rates of glycine and glycyl-glycine.** By H. NEWEY and D. H. SMYTH. *Department of Physiology, University of Sheffield*

Newey & Smyth (1959*a*) showed that if dipeptides are present in the intestinal lumen they enter the blood stream predominantly as amino acids. Later work (Newey & Smyth, 1959*b*) showed that glycyl-glycine could enter the mucosal cells and was hydrolysed intracellularly. Since there is considerable evidence for the existence of a special mechanism for intestinal transfer of amino acids, the question arose whether there is also a special mechanism for the dipeptide transfer. Experiments therefore were carried out to compare the rate of entry of glycine and glycyl-glycine into the mucosal cells.

The *in vitro* preparation was the sac of everted rat small intestine (Wilson & Wiseman, 1954). Sacs were filled with 1 ml. bicarbonate saline containing 500 mg glucose/100 ml., and were incubated in 50 ml. of the same solution to which was added either glycine or glycyl-glycine (15·1 mM). After 30 min the amount of glycine present in the mucosal and serosal fluids and in the intestinal wall was estimated. Results showed that the rate of entry of glycyl-glycine into the mucosal cells was approximately the same as rate of entry of glycine. Anaerobic conditions and 2:4-dinitrophenol ($2 \times 10^{-4}$ M) greatly reduced the entry of both glycine and glycyl-glycine. These conditions did not affect peptidase activity in homogenates.

The *in vivo* experiments were carried out with the preparation of Sheff & Smyth (1955). When solutions of glycine or glycyl-glycine (37·8 mM) were circulated through the intestinal lumen of the anaesthetized rat for 30 min they disappeared from the lumen at approximately the same rate.

These experiments suggest that glycyl-glycine is absorbed by a mechanism other than diffusion, and hence raise the question whether there is one mechanism which can transfer both glycine and glycyl-glycine. Since anaerobic conditions interfere with intracellular hydrolysis but do not inhibit peptidase activity, there is probably a mechanism for entry of glycyl-glycine into the cell, and this mechanism functions only under aerobic conditions. The mechanism for amino-acid transfer, postulated by Gibson & Wiseman (1951), may be located at the mucosal border of the cell and be capable of dealing with both peptides and amino acids. This

mechanism may be only for entry of these substances, and there may be another mechanism for movement of the amino acids against a concentration gradient. The possibilities of the site of the mechanism are analogous to those suggested by Newey, Parsons & Smyth (1959) for glucose. Dipeptides may thus play an important part in the absorption of dietary protein, even though they do not subsequently enter the blood stream.

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**The effects of tyramine on the isolated guinea-pig heart.** By P. A. Nasmyth. *Department of Pharmacology, St Mary's Hospital Medical School, London, W. 2*

Burn & Rand (1958) have suggested that in the intact animal tyramine normally acts by releasing noradrenaline or adrenaline from the store in the artery wall. By contrast, the effects of tyramine on the isolated guinea-pig heart perfused with Krebs's solution by Langendorff's method would seem to be of interest.

The increase in amplitude of the beat in response to regular injections of 5–10 $\mu$g of tyramine, given at 5 min intervals, gradually diminishes and finally disappears whether the drug is injected at regular intervals or not. When the response to tyramine has disappeared, the injection of 10 $\mu$g of noradrenaline causes it to reappear for a period of about 20 min. The inclusion of tyramine in the perfusion fluid at a concentration of $5 \times 10^{-6}$ g/ml. for a period of 30 min results in a disappearance of the response to injected tyramine, both during the perfusion of tyramine and after the drug is removed from the perfusion fluid.

The pressor amine content of hearts perfused in these various ways was estimated in terms of noradrenaline (ng/g wet weight) with the following results:

| Perfusion fluid | Duration of perfusion | Response to tyramine | Pressor amines (ng/g ± S.E.) | No. of hearts |
|---|---|---|---|---|
| Krebs's solution | 30 min | Present | 552 ± 61 | 11 |
| Krebs's solution + tyramine $5 \times 10^{-6}$ g/ml. | 30 min | Absent | 602 ± 57 | 10 |
| Krebs's solution | 3–5 hr | Absent | 559 ± 41 | 10 |

The immediate effect of including reserpine $10^{-6}$ g/ml. in the perfusion fluid was to potentiate the response to injected tyramine. This effect

coincides with the period during which one might expect reserpine to be releasing noradrenaline from stores in the heart. Following upon the short period of potentiation, the responses to tyramine gradually diminish and eventually disappear. These results suggest that the loss of response to tyramine in the isolated guinea-pig heart and its restoration with doses of noradrenaline may be related not so much to intracellular noradrenaline as it is to noradrenaline in the extracellular fluid.

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**The effect of stimulating the vagus nerve on the mechanical events of the heart.** By S. K. BROCKMAN, J. P. GILMORE, R. J. LINDEN,* J. H. MITCHELL and S. J. SARNOFF. *Laboratory of Cardiovascular Physiology, National Heart Institute, Bethesda, Maryland, U.S.A.*

In the anaesthetized dog with the chest open, heart rate, pressure in the aorta and the left atrium, and mean blood flow in the aorta were recorded simultaneously. The heart rate was maintained constant by stimulating the left atrium. Both vagi were divided in the neck.

With the heart rate constant, stimulation of the peripheral end of one vagus nerve resulted in a diminution in cardiac output, a fall in aortic blood pressure and a rise in mean atrial pressure; these effects were abolished by atropine. These results can be interpreted as a negative inotropic effect of the vagus on the ventricle. That there is such a negative inotropic effect was suggested by Peterson (1950).

However, other explanations are possible. There might have been a change in the extensibility of the left ventricular muscle. By recording changes in length of a segment of myocardium as described previously (Linden, Mitchell & Sarnoff, 1960) it was shown that there was no change in the relationship between end-diastolic pressure in the ventricle and myocardial segment length when the vagus nerve was stimulated. Thus it may be inferred that there was no change in extensibility of the ventricular muscle.

Another possibility is that during stimulation of the vagus nerve changes in mean atrial pressure do not indicate similar changes in ventricular end-diastolic pressure. In experiments in which the pressure in the ventricle also was recorded, stimulation of the vagus nerve resulted in a decrease in end-diastolic pressure in the ventricle although there was a simultaneous increase in mean atrial pressure. Further, the relationship

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between stroke work (mean aortic pressure × stroke volume) and end-diastolic pressure in the ventricle was examined over a range of end-diastolic pressures by repeated infusion and bleeding; this relationship was unaltered during stimulation of the vagus nerve. Therefore there was no negative inotropic effect on the ventricle.

In experiments in which heart block was produced by section of the bundle of His and in which the atrial rate was kept constant it was shown that the 'a' wave of the atrial pressure pulse (which results from atrial contraction) was diminished during stimulation of the vagus nerve. This result confirms the suggestion of McWilliam (1888) that stimulation of the vagus nerve results in a diminished atrial contractility.

It is suggested that, with the heart rate constant, the sole effect on the mechanical events of the heart of stimulation of the vagus nerve is to decrease atrial contractility. As a result of the decreased atrial contraction the ventricle is less filled during ventricular diastole. Thus atrial volume and mean pressure rise, and cardiac output and arterial blood pressure fall.

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**The secretory response to discontinuous infusions of histamine in the dog.** By E. H. L. HARRIES. *Department of Physiology, University of Birmingham*

The use of 'double' histamine tests for assessing inhibitors of gastric acid secretion is well established. Histamine has been given either as a single subcutaneous injection or by continuous intravenous infusion, both in anaesthetized and unanaesthetized animals, and in man. During the course of experiments designed to measure the effects of sympathomimetic amines on gastric secretion, it was noticed that the secretory responses to identical infusions of histamine were often irregular.

Unanaesthetized dogs with simple gastric fistulae were used. Histamine acid phosphate (0·75 $\mu$g/kg/min) was administered by continuous intravenous infusion. By giving the histamine infusion for 35 min followed by a period of 35 min between infusions, it is possible to perform experiments on two dogs simultaneously with the same injection apparatus. Both dogs can be given five infusions each within 6·5 hr.

As may be seen in Fig. 1, the response to a second infusion of histamine is often far greater than the response to any of the other infusions on the

same day. In the experiments where the dogs received five infusions, the mean amounts of total acid (m-equiv) secreted were respectively

2·94 3·98 3·00 2·86 2·75

In all the experiments *t*-tests have been performed on the column of differences between the first and second, and also between the second and third responses. The probability of the high second response being due to chance is less than 5%.

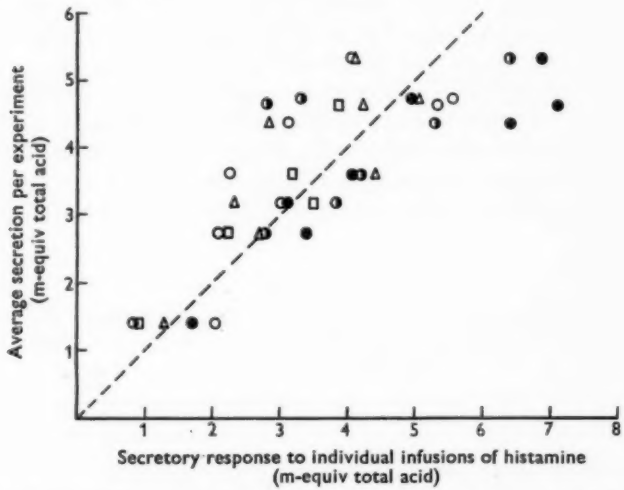


Fig. 1. Secretory response to repeated equal infusions of histamine on the same day. Dotted line represents the mean response. First response = ○; second response = ●; third response = △; fourth response = ◑; fifth response = □.